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AVN-211, Novel and Highly Selective 5-HT_6 Receptor Small Molecule Antagonist, for the Treatment of Alzheimer's Disease

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ABSTRACT

Within the past decade several novel targets have been indicated as key players in Alzheimer-type dementia and associated conditions, including a 'frightening' memory loss as well as severe cognitive impairments. These proteins are deeply implicated in crucial cell processes e.g. autophagy, growth and progression, apoptosis and metabolic equilibrium. Since recently, 5-HT_6R has been considered as one of the most prominent biological targets in AD drug therapy. Therefore, we investigated the potential pro-cognitive and neuroprotective effects of our novel selective 5-HT_6R antagonist, AVN-211. During an extensive preclinical evaluation

the lead compound demonstrated a relatively high therapeutic potential and improved selectivity towards 5-HT_6R as compared to reference drug candidates. It was thoroughly examined in different *in vivo* behavioral models directly related to AD and showed evident improvements in cognition and learning. In many cases, the observed effect was considerably greater than that determined for the reported drugs and drug candidates, including memantine, SB-742457 and Lu AE58054, evaluated under the same conditions. In addition, AVN-211 showed a similar or better anxiolytic efficacy than fenobam, rufinamide, lorazepam and buspirone in an elevated plus-maze model, elevated platform and open field tests. The compound demonstrated low toxicity and no side effects *in vivo*, an appropriate pharmacokinetic profile as well as stability. In conclusion, AVN-211 significantly delayed or partially halted the progressive decline in memory function associated with AD that makes it an interesting drug candidate for the treatment of neurodegenerative and psychiatric disorders. The advanced clinical trials is currently under active discussion and in high priority.

KEYWORDS: 5-HT_6R, antagonists, Alzheimer's disease, selectivity, preclinical study, pharmacokinetics

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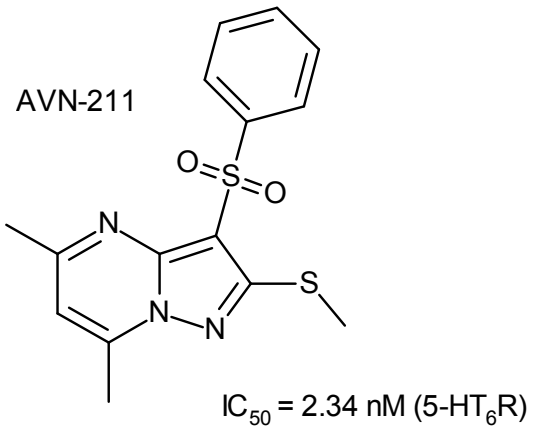
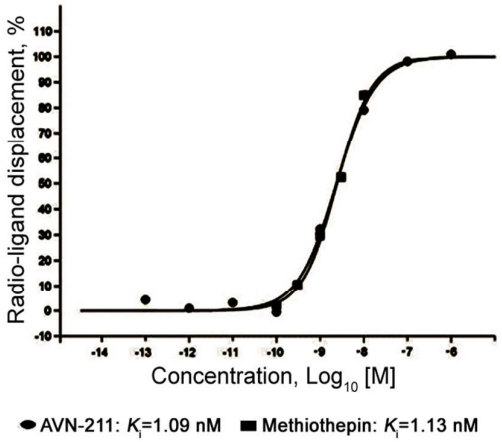




INTRODUCTION

Alzheimer's disease (AD) is, undoubtedly, the most common and progressive form of dementia (up to 60–70% of cases) diagnosed particularly among the elderly, and 60% of these people live in Western, low- and middle-income countries. Currently, according to World Health Organization, more than 35 million alzheimer's report numbers exist.[1] In Western hemisphere, the prevalence of AD is roughly estimated around 1% among the subjects aged 60–65, however this number increases up to 35% in people aged over 85. As a result, above 5.5 million people are currently diagnosed with AD in United States. Generally, the disease is accompanied by severe memory loss and disorientation, social detachment, significant loss of independence, and ultimately leads to lethal outcome within 3−9 years since the diagnosis has been confirmed and this frightening statistics is actually expected to get much worse. Therefore, without radical breakthroughs in the field of innovative treatment and care of AD the associated costs are indeed expected to grow dramatically. During the past decade, considerable advances have been made towards the understanding of tangled nature of this pathology and neurodegeneration associated with this disease. However, despite the outstanding achievements in this field, an effective cure strategy is still wanting. With regard to drug treatment, many painstaking attempts have been made resulting in several small-molecule compounds with promising activity against AD (Table 1).

Table 1. Representative examples of molecules targeted against AD

Drug Name	Mechanism of action	Originator	Highest Phase	Ref
DMXB-Anabaseine	nicotinic α_7 partial agonist	CoMentis	Phase II	2
Ladostigil	butyrylcholinesterase	Avraham	Phase II	3

	inhibitor, MAO-B/A inhibitor			
VX-745	MAPK p38 inhibitor	EIP Pharma	Phase II	4
ZSET-1446	T-type calcium channel activator	Sonexa Therapeutics	Phase II	5
Latrepirdine	multi-targeted	Pfizer	Phase III	6
Lu AE58054	5-HT_6R antagonist	Lundbeck	Phase III	7,8
SB-742457	5-HT_6R antagonist	GSK	Phase II	9
Encenicline	α_7 agonist	FORUM Pharmaceuticals	Phase III	10
AC-1204	AC-1202 analogue (Dietary Supplement)	Accera	Phase II/III	11
Verubecestat	BACE1 inhibitor	Merck & Co.	Phase III	12
AZD-3293	BACE1 inhibitor	AstraZeneca	Phase II/III	13
Leuco-MTx	MAO inhibitor, Tau aggregation inhibitor, NO production inhibitor	TauRx Therapeutics	Phase III	14

Monoamine oxidases (MAO); p38 mitogen-activated protein kinases (MAPK p38); 5-hydroxytryptamine (serotonin) receptor 6 (5-HT_6R); Beta-secretase 1 (BACE1)

Particularly, it has been proposed that Latrepirdine (Dimebon) may modulate several targets including voltage-gated calcium channels, mitochondrial permeability pore transition, or several neurotransmitter receptors.[15–21] However, this type of therapy is specifically targeted on symptomatology (BPSD) and improvements in the quality of life thereby providing *per se*

temporary effect. Recent findings have indicated several novel prominent targets deeply implicated in AD neuropathology, including GLP-1R (AD is increasingly regarded as type 3 diabetes),[22,23] proteins that regulates autophagy (e.g. PINK1|2,[24] mTOR,[25] TLRs,[26] SUMO1,[27] Wnt/β-catenin,[28] ACAT1,[29] TREM2)[30] and pro-survival intracellular pathways (repair/regenerative processes) as well as γ|β-secretase,[31,32] growth factors and serotonin receptors (improvements in memory and/or cognition).[33,34] It should be noted that 5-HT_6R was tentatively suggested to be involved in autophagy regulation as well via mTOR signaling pathway.[25,35,36]

In recent years the important role of 5-HT_6R and 5-HT_6R antagonists in AD neuropathology has been abundantly highlighted [for review see: [34,37–41]]. Despite several "unsuccessful" stories with 5-HT_6R antagonists in clinics, including the one with Dimebon, novel small-molecule compounds possessing high activity against this receptor subtype are currently undergoing clinical evaluation related to AD neuropathology. For instance, Lu AE58054 (*vide supra*), PF-05212377 (Phase II, Pfizer),[42] SUVN-502 (Phase II, concomitant therapy, Suven Life Sciences),[43] as well as our parent compounds, including AVN-101[44] and AVN-322[8] (Phase I/II, Alla Chem.). Interestingly, some leading pharmaceutical companies have made the decision to re-profile their anti-schizophrenia molecules towards AD trials, since selective 5-HT_6R antagonists have recently been considered "behind-the-scenes" as prospective drug candidates against AD.

Here we describe the pharmacokinetic features, activity, efficiency as well as toxicity profile of AVN-211. We clearly demonstrated that this compound is a novel highly effective (single digits nM) 5-HT_6R antagonist with dramatically improved selectivity as compared to other reported molecules targeted against the title receptor, including the most advanced in clinics Lu-AE58054 and SB-742457. AVN-211 (4 and 8 mg, 2-6 weeks) has recently been evaluated in Phase II clinical trial at Avineuro for the treatment of schizophrenia. Based on the obtained results, we made a decision to test the compound within an extensive clinical trial

following the parent indication as well as re-profile it against AD by analogy to other 5-HT_6R antagonists Lu-AE58054 and SB-742457. The results of a comparative preclinical trial disclosed herein have revealed AVN-211 as a promising drug candidate, which has demonstrated an improved PK profile and efficiency in different *in vivo* AD-related models as compared to other therapeutics including selective 5-HT_6R antagonists, Memantine (NMDAR antagonist) and Tacrine (AChE inhibitor). Considering a prominent role of 5-HT_6R and selective 5-HT_6R antagonists in Alzheimer's neuropathology, AVN-211 can be reasonably regarded as novel drug candidate with an outstanding therapeutic potency against AD.

MATERIAL AND METHODS

Due to severe limitations in the available paper space, the detailed experimental protocols, procedures as well as lab. tools are properly discussed in *supplementary materials (SM)*. Below, we provide only a brief description.

Screening

HTS and follow up studies were performed using a recombinant human 5-HT_6R stably expressed in HEK293 cells in a T-Rex™ System, a tetracycline regulated mammalian expression assay kit. All the reagents used were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Radiochemicals were purchased from Amersham Radiochemicals (GE Healthcare Life Sciences, Pittsburgh, PA). Membrane preparations from cells either transfected with or endogenously expressed receptors and enzymes were obtained from MDS Pharma Services and Eurofins Sc. (Taipei, Taiwan). SK-N-SH cells were from American Type Culture Collection (Manassas, VA). Human recombinant tetracycline-inducible HEK293 5-HT_6R-T-Rex cells were obtained from Etogen Scientific (San Diego, CA). Competitive radio-ligand binding assay for AVN-211 towards a broad panel of therapeutically significant targets has been

performed in MDS Pharma Services and Eurofins Sc. following the procedure described in the paper by Okun et al. [47]

Stability in human liver s9 fraction

Analysis of AVN-211 stability in the presence of human liver s9 fraction (CellzDirect, USA) was performed in according to the procedure reported in manufacturer guidelines.[48]

Permeability

Permeability of AVN-211 was determined using Caco-2 cells (ATCC cat № HTB-37). Assay was performed according to Caco-2 plate manufacturer guidelines.[49]

Interaction with P-gp

Two different approaches, luminescent ATPase assay with P-gp-Glo System and Caco-2 monolayer efflux assay, were used to study P-gp/AVN-211 interaction. The first assay has been applied to detect the effects of compounds on recombinant human P-gp in a cell membrane fraction. The assay relies on the ATP dependence of the light-generating reaction of firefly luciferase. MultiScreen Caco-2 Assay System (Millipore Corp., USA) was used for the monolayer efflux studies. Caco-2 cells are morphologically and functionally very similar to intestinal barrier cells and are frequently utilized as *in vitro* permeability model. The cells also express a variety of transporters including P-gp, thus may be used in P-gp-mediated transport studies. Particularly, the effect of AVN-211 on Rhodamine^{123} transport has been estimated.

Interaction with CYPs

The interaction of AVN-211 with CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Invitrogen Vivid® CYP450 Screening Kits, Cat. no. P2863, P2859, P2864, P2972,

P3021, P2856) and CYP2B6 (BD Biosciences P450 Inhibition Kit, Cat. no. 459220) was studied in full accordance with the manufacturer's protocols.[50]

Animals

Mice (17-33 g, 8-9 wk old) and rats (150-320 g, 13-14 wk old) were obtained from the State Scientific Center (GU NC) of Biomedical Technologies of the Russian Academy of Medical Sciences (RAMS) "Andreevka" (Moscow reg.). Studies with Rhesus Macaques (*M. mulatta*) were performed at the Research Institute of Medical Primatology (Adler, Russia). Monkeys were recommended by the regulatory body as non-rodent species for a number of reasons: data on CNS drug candidates have usually better translation from primates to humans; it is assumed that primates have more "human-like" pattern of serotonin receptors expression.

In vivo PK study

Pharmacokinetic properties of the compound were evaluated using 24 male rats of WAQ strain. Oral bioavailability of AVN-211 was calculated based on the obtained results as a ratio of $AUQ_{0\rightarrow t}$(p.o.)/$AUQ_{0\rightarrow t}$(i.v.). We also investigated the PK features of AVN-211 in rat brain. The studies were performed on male Wistar rats. Preliminary escalating dose study was conducted in male Wistar rats (300–330 g). Animals were kept in standard laboratory conditions. Animals were divided into 6 separate groups (3 animals per each group). Three groups were administered with AVN-211 *per os* in 6 doses: 1, 3, 10, 20, 50 and 100 mg/kg. The remaining groups were administered with AVN-211 intravenously in 3 doses: 1, 3 and 10 mg/kg. After the injection, animals were allowed to rest for 60 minutes. Then, animals were sacrificed and plasma samples were collected and subsequently analyzed for AVN-211 concentration using LC-MS/MS API2000. SHK mice were also used to estimate PK features of AVN-211. The BBB permeability of AVN-211 was estimated in rats (10 mg/kg, p.o.). Five rhesus monkeys were used for PK

evaluation. Animals were not recruited in other studies (e.g. as a placebo-control group) for at least 2,5 months prior the PK trials. Animals were kept in cages, feeding was performed with usual balanced fodders, eggs, vegetables and fruits. Clinical status of animals and their behavior were normal. Animal IDs, weight, birthday as well as the route of administration and doses of the tested compounds are presented in SM. Biodistribution of the studied compound was examined in rats using [H^3]-AVN-211 (10 mg/kg). In this experiment, 18 male rats (180–200 g, WAQ strain) were used. The animals were kept in standard laboratory conditions. Maintenance and care were in accordance with the Guide for the Care and Use of Laboratory Animals.[51]

In vivo efficacy

The rats (Wistar and Sprague-Dawley, 160–300 g, 13–14 wk old) and mice (BALB/c and SHK, 18–32 g, 8–9 wk old). Before the studies, the animals were quarantined for 7 days. During this period, the animals were monitored for the presence of possible diseases. They were housed in standard plastic cages under controlled conditions: temperature 19–25 °C, humidity 50–70%, 12:12 h light-dark cycle, concentration of carbon dioxide <0.15% (vol.), concentration of ammonia <0.001 mg/L. The animals were monitored at least once daily (or 1 h after subacute injections). The results of monitoring were traced in a laboratory register. The specificity of the subjects used are listed in Table 9 (*see below*).

Most of the reference compounds, including commercially available Sibutramine, Haloperidole, Lorazepam, Fenobam, Rufinamide, Buspirone, Memantine and Tacrine were used in consistent doses and the doses were chosen based on the literature and tests performed at the lab. Particularly, SB-742457 and PRX-07034 were tested in different doses (titrated) in the same fashion as it was done for AVN-211. It is important to emphasize that the most efficacious dose of each compound varied in different species of rodents (for example there was clear difference between efficacy in SHK and BALB/C mice) and also it varied depending on the challenge given

to animals (MK-801 vs. scopolamine). The most efficacious dose of SB-742457 or PRX-07034 is cited in the manuscript (*vide infra*). The details of titration and corresponding dose range are presented in SI.

hERG channels inhibition

Potassium current was measured using the patch clamp technique in Molecular Devices' Patch Express 7000A following the procedure described in SM.

Toxicity and side-effects

LD_{50}, MTD and 14-days toxicity of AVN-211 were investigated in SHK strain male mice (20–25 g). We estimate 29- and 30-days toxicity of AVN-211 in 80 Wistar rats and rabbits, respectively. Long-term 180-days toxicological study was also performed in 80 Wistar rats (8-weeks old). In addition, six healthy Rhesus Macaques (Macaca Mulatta, 3 males and 3 females) were used to determine 30-days toxicity of AVN-211. Pathomorphological examination, including histopathology and macroscopic study, was performed in mice (10 males and 10 females) after AVN-211 administration *per os* in the dose of 3 mg/kg. Rabbits and Wistar rats were also used under the same conditions. *In vitro* mutagenicity was estimated using Ames MPF Test, while mutagenicity *in vivo* was analyzed in C57BL/6 mice (18–20 g, 8–12 weeks old). All the details of the procedures mentioned above are conveniently described in SM with corresponding tables and figures.

Statistical analysis

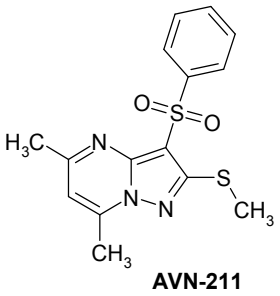
All data were fed into Excel 2003 spread sheets. Subsequent statistical analyses were performed using Statistica 6.0 software. The obtained results are presented as mean ± standard

error of the mean (S.E.M.), unless otherwise stated. A p-value less than 0.05 was considered statistically significant. LD_{50} was calculated from the mortality rate table according to the method of Finny using BioStat 2006 program.

RESULTS

Molecule

AVN-211 (5,7-dimethyl-2-(methylthio)-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidine, Figure 1) is a highly selective 5-HT_6R antagonist, with K^b_i=1.09 nM (competitive binding assay), K^f_i=0.83–1.97 nM (functional assay)[45] obtained by a convenient 3-step synthetic route in high yield (up to 90%) using common chemical reactions.[46]

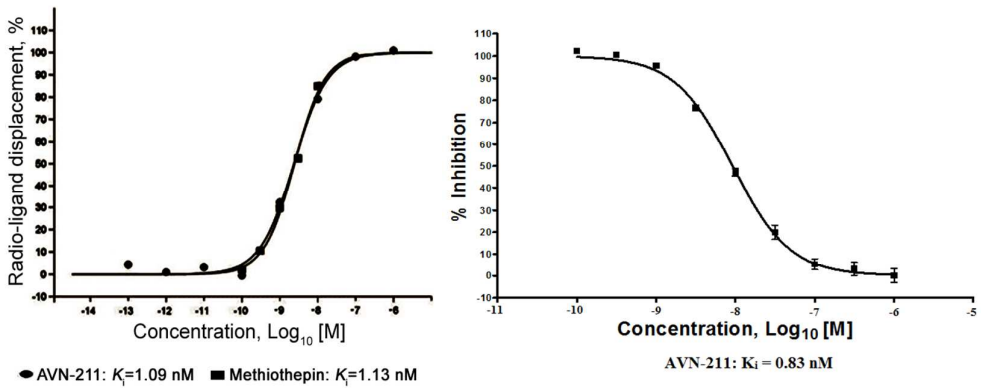


MW=333.44
LogD=2.37 (pH=7.4)
LogP=1.21 (pH=4)
LogP=2.37 (pH=9.5)
logSw=-6.12
HBA=4
HBD=0
RBN=1
PSA= 98.0

Figure 1. Structure of AVN-211 and key features.

Screening and selectivity

Historically, more than 100K compounds available within ChemDiv proprietary library have been screened on a panel of GPCRs reportedly involved in pathophysiology of AD. Disclosed hits were then tested in *in vitro* and *in vivo* behavioral models and further optimized on a smaller panel of GPCRs. Target-based HTS and profiling have revealed AVN-211 as a highly potent and selective 5-HT_6R antagonist (Figure 2): IC_{50}=2.34 nM, K^b_i=1.09 nM and EC_{50} = 7.1–16.0 nM, K^f_i = 0.83–1.97 nM.



a **b**

Figure 2. Activity of AVN-211: (**a**) competitive displacement of radio-labeled [^{3}H]LSD from 5-HT$_6$R expressed in the cell membrane fraction; (**b**) dose-dependent inhibition of cAMP production induced by serotonin in HEK293 cells transfected with functional human 5-HT$_6$R.

AVN-211 specificity profile was accurately determined using a wide panel of various proteins, including GPCR family, ion channels, and transporters (Figure 3, Supplementary Table 1). As clearly shown in Figure 3 (and Table S1 in SI), AVN-211 exhibited 5K-fold higher 5-HT$_6$R selectivity over other 65 receptors, enzymes, and ion channels except 5-HT$_{2B}$R sub-type (IC^f_{50}=0.196 μM, EC_{50}>10 μM). Within the 5-HTRs group the compound showed about 100-fold 5-HT$_6$R selectivity.[52]

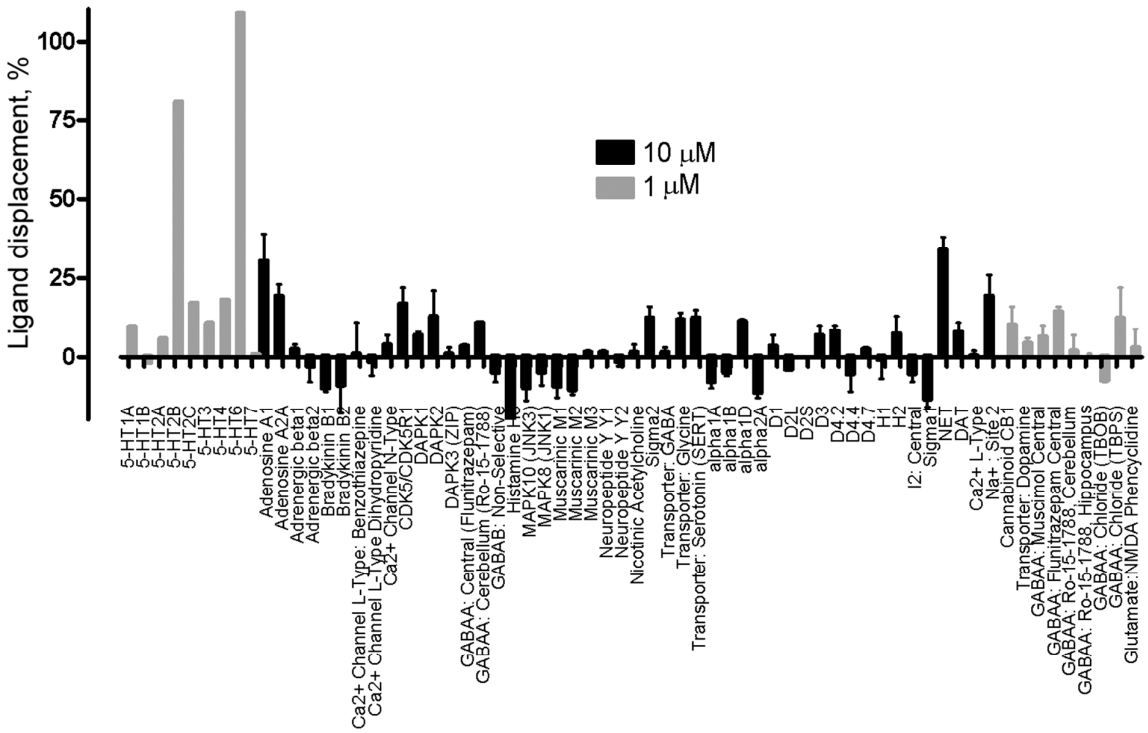


Figure 3. Selectivity profile of AVN-211 (at 1 and 10 μM) towards 66 targets determined in the radio-ligand binding assay (the detailed data is presented in SM, Supplementary Table 1).

One of the most crucial point of AVN-211 is an outstanding selectivity exclusively towards 5-HT_6R[53] as compared to known antagonists which has recently been reported. For instance, the most advanced drug candidates Lu-AE-58054 and SB-742457 demonstrated rather moderate 5-HT_6R selectivity that was respectively more than 100- and 50-fold lower than the selectivity of AVN-211 keeping a comparative target affinity. Indeed, the selectivity profile of Lu-AE-58054 (K_i=0.83 nM, 5-HT_6R) determined in more than 80 binding as well as in 20 enzymatic assays indicated >50 fold selectivity except for the adrenergic α_{1A} (K_i=21 nM) and α_{1B} (K_i=22 nM) receptors. Lu-AE-58054 also showed nanomolar activity towards 5-HT_{2A}R (K_i=83 nM) and 5-HT_{2C}R (K_i=250 nM).[54] With regard to SB-742457 (pK_i=9.63, 5-HT_6R, binding assay), it showed >100-fold increase in the target activity over 85 other proteins of different types.[55] However, in a radio-ligand binding assay, at the concentration of 1 μM, it demonstrated a multi-targeted profile, including the core 5-HT_6R (101% inhibition) as well as 5-HT_{1A}R (73%),

5-HT_{1B}R (102%), 5-HT_{2A}R (99%), 5-HT_{2B}R (99%), 5-HT_{2C}R (95%), 5-HT_7R (84%) receptor subtypes. Moreover, we have demonstrated that SB-742457 was quite active against adrenergic α_{1B} (50%), α_{2A} (51%), β_1 (90%) and β_2 (83%), dopamine D_{2L} (76%) and D_{2S} (76%), imidazoline I_2 central (60%) and σ_1 (57%) receptors, calcium L-type benzothiazepine (66%) and dihydropyridine (55%) and sodium site 2 (55%) channels, as well as NET (78%) and DAT (85%) transporters.[56]

Affinity to benzodiazepine receptor

In vivo evaluation revealed AVN-211 as a potent anxiolytic (*vide infra*). In order to assess the mechanism of anxiolytic action, an additional radioligand displacement assay was performed with peripheral benzodiazepine receptor. As clearly shown in Figure 3, AVN-211 did not interact with benzodiazepine receptor, thus it's anxiolytic effects observed in 3 different *in vivo* models were not likely to be related to peripheral benzodiazepine receptors.

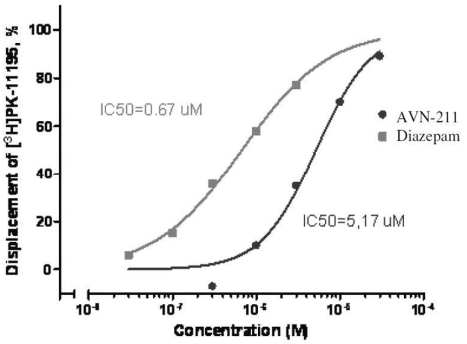
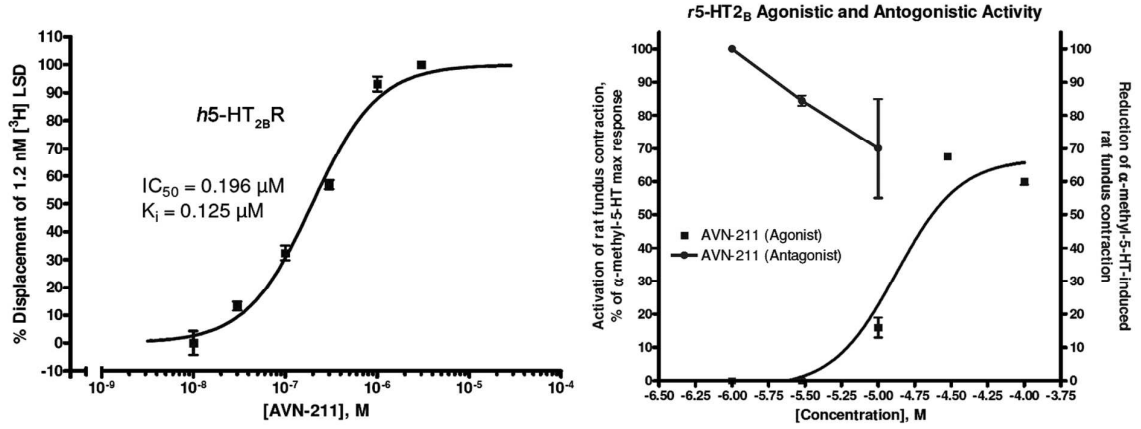


Figure 3. Radioligand displacement assay with peripheral benzodiazepine receptor

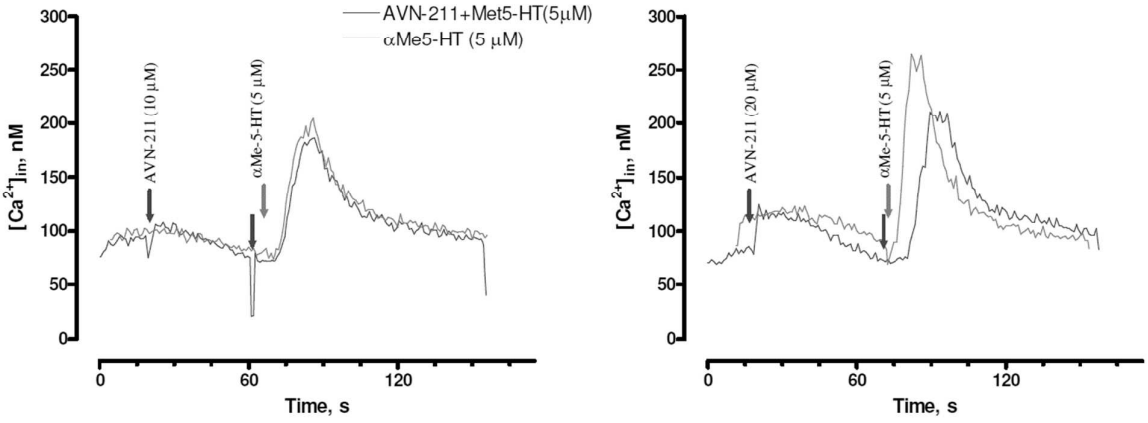
Interaction with 5-HT_{2B}R

In some cases, the 5-HT_{2B}R agonism has been shown to be implicated in a cardiac impairment.[57] Our data, showing that AVN-211 binds to the receptor, has made it necessary to study its effect on 5-HT_{2B}R in more details to address its potential liability. Thus, in a radioligand competitive binding assay, AVN-211 showed K_i values of 0.125 μM (Figure 4a), which was 60-fold higher than that observed for 5-HT_6R. AVN-211 was tested in a functional

tissue assay, rat fundus contraction, both as an agonist (induction of fundus contractions) and antagonist (inhibition of α-methyl-serotonin-induced contractions). The data depicted in Figure 4b shows that AVN-211 is a partial agonist of the Rat receptor with EC_{50} around 13 μM, which is about 100-fold higher than its antagonistic activity against the same receptor, IC_{50}=163 nM (cell-based functional assay). Additional functional aequorin kinetic assays (Euroscreen) were performed to measure possible AVN-211 agonism of human 5-HT_{2B}R. AVN-211 was used in two concentrations – 10 μM and 20 μM (Figure 4c) and did not show any agonism at either concentration. Dose-dependence curve of AVN-211 was also obtained using aequorin functional assay (Figure 5).



a b



c

Figure 4. (**a**) Dose-dependent displacement of [^{3}H]LSD from 5-HT$_{2B}$ receptor with AVN-211; (**b**) dose-dependence agonist/antagonist curve of AVN-211; (**c**) intracellular calcium dynamics in AVN-211-treated cells. AVN-211 was used in two concentrations - 10 μM (*left*) and 20 μM (*right*)

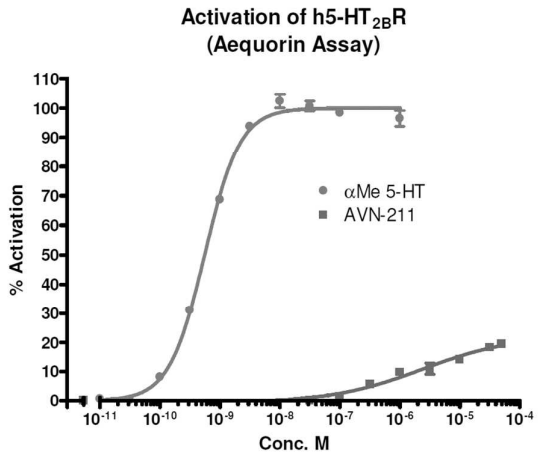


Figure 5. Activation of *h*5-HT$_{2B}$R by AVN-211

The aforementioned data clearly indicates that AVN-211 is a highly selective and specific antagonist of 5-HT$_6$R with very good safety window.

In vitro ADME features

Analysis of AVN-211 (17 μM) stability in the presence of human liver s9 fraction (Figure 6) showed $t_{1/2}$ value of more than 2 h. The compound reached the maximal degradation of only 5% of the initial concentration. Thus, AVN-211 was quite stable in the presence of s9 human liver fraction.

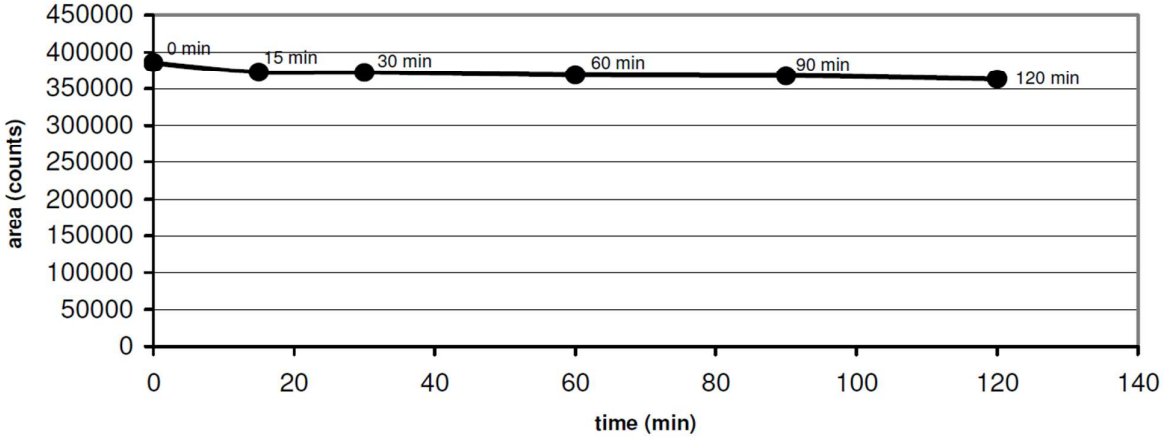


Figure 6. Dynamics of AVN-211 concentration during incubation with human s9 fraction

Analysis of AVN-211 (500 ng/mL) stability in the human plasma showed only 16% reduction in the original compound concentration during the first 30 min of the incubation. AVN-211 binding to human plasma proteins was assessed using the Pierce rapid equilibrium device (RED) system. According to the obtained data, 87.8% of AVN-211 was bound to human plasma proteins. The same binding was observed in rat plasma (PPB=88.2%). Permeability of AVN-211 was estimated in Caco-2 cells (*for details, see SM*). It was demonstrated that AVN-211 has a relatively high permeability in Caco-2 assay conditions (Papp(10^{-6} sm/s) = 41.4; LC/MS/MS).

P-glycoprotein is an ATP-dependent efflux transporter that affects the absorption, distribution and clearance of a variety of molecules. The modulation of P-gp function through inhibition or stimulation can lead to drug-drug interactions and alter pharmacokinetics, efficacy, safety and target organ specificity. Thus, the *in vitro* P-gp assays are used in drug development to identify the potential interaction of drug candidates with P-gp. Compounds could be classified as P-gp substrates, inhibitors, inducers or have no effect on P-gp function. It is well known that a single assay format is unable to provide a complete and definite information about P-gp-related effects of compounds. Therefore, we used two different approaches (*vide supra*). Thus, during the first assay AVN-211 stimulated P-gp-ATPase activity in a concentration-dependent manner at concentrations above 10 μM, while at 45 μM the compound showed the same effect as

verapamil, a Pgp-inhibitor used as a positive control (see SI). Stimulation effect was only seen in the concentrations exceeding 10 μM, which were much higher than therapeutic concentrations. Therefore, it can be concluded that AVN-211 would not affect P-gp-ATPase activity in therapeutic conditions.

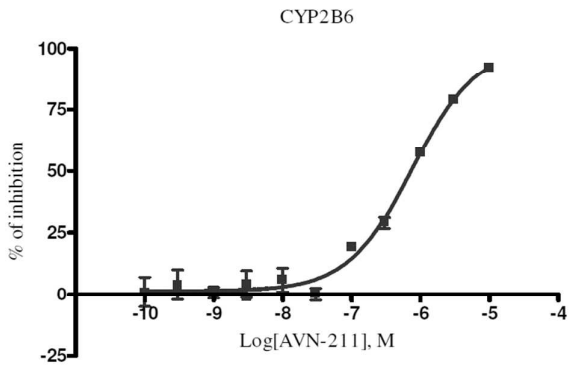
AVN-211 showed good passive permeability in MultiScreen Caco-2 assay system. There was no difference between transport rates in apical to basolateral and basolateral to apical directions. The Papp B-A/A-B ratio was about 1.0 at the whole concentration range. This finding suggests that AVN-211 does not undergo efflux and is not transported by P-gp. However, there is an opinion that the monolayer efflux assays tend to be insensitive to highly permeable compounds (Papp A-B > 30×10^{-6} cm/s), thereby yielding false negative results.[58] The compound may not be effluxed by P-gp, but could affect the transport of other P-gp substrates due to the complexity of the possible molecule–protein interactions. Thus, we used Rhodamine-123 (Rh123), a well-characterized and easily detectable P-gp substrate to estimate this effect under the analogue conditions. According to the results obtained in both tests: AVN-211 was negative in Caco-2 monolayer efflux assay, but stimulated P-gp ATPase activity at concentrations above 10 μM. In addition, a weak decrease in Rhodamine-123 efflux was observed in the presence of 20 μM AVN-211. Thus, AVN-211 can interact with P-gp at micromolar concentration. It is unlikely to be transported by P-gp or its efflux was not detected due to its very high permeability.

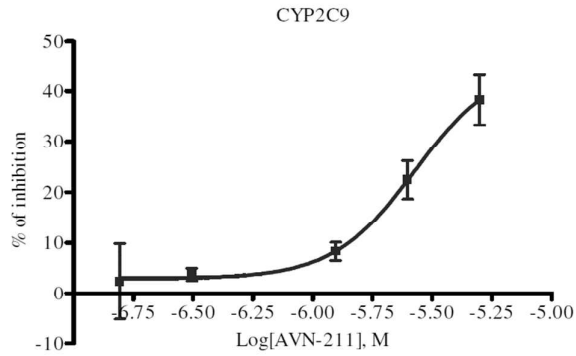
The effect of AVN-211 on CYP450 family was investigated following the procedure described above. The obtained results are presented in Table 2 and Figure 7. As clearly shown below, AVN-211 interacts with CYP2B6, CYP2C9 and CYP2C19. The PK profile of AVN-211 and its metabolites have been estimated in humans at different oral doses and schedule. Thus, we revealed several key metabolically active points, including the sulphur atom (*S*-oxidation), methyl groups attached to pyrimidine moiety (monooxygenation) and phenyl ring (*p*-position,

monooxygenation). The details of this study will be discussed properly within the follow up paper.

Table 2. Results of the CYP450 inhibition assays

CYP isoform	Max% inh	SD	IC_{50}, pM	Hillslope	Conclusion
1A2	-	-	-	-	not active
2B6	95.5	1.6	0.76	0.93	active
2C9	43.7	4.7	2.68	2.515	active
2C19	36.26	0.83	8.08	1.742	active
2D6	-	-	-	-	not active
2E1	-	-	-	-	not active
3A4	-	-	-	-	not active





a **b**

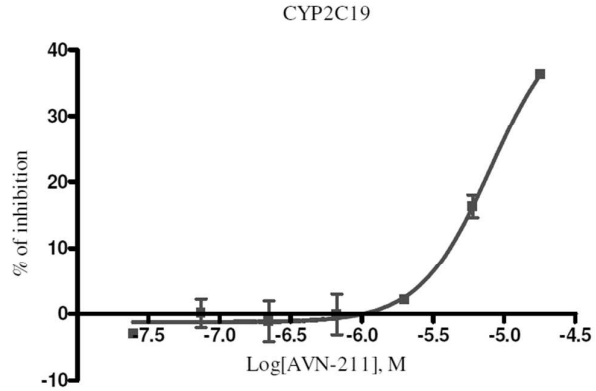


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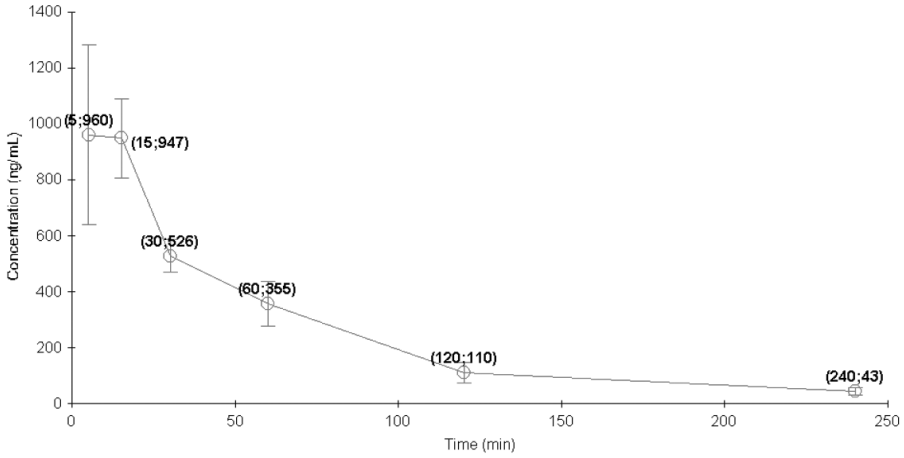
Figure 7. CYP inhibition curves for AVN-211: IC_{50}: 0.75, 2.68 and 8.08 μM (partial inhibition), respectively

In vivo PK profile

Figure 8 shows concentrations of AVN-211 (ng/mL) in rat blood plasma after a single intravenous injection (i.v.) in the dose of 5 mg/kg and 10 mg/kg (p.o.). Pharmacokinetic parameters are listed in Table 3.



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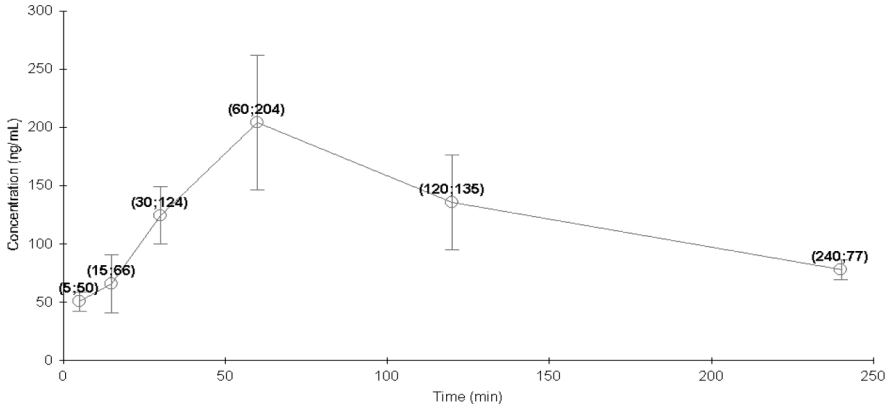

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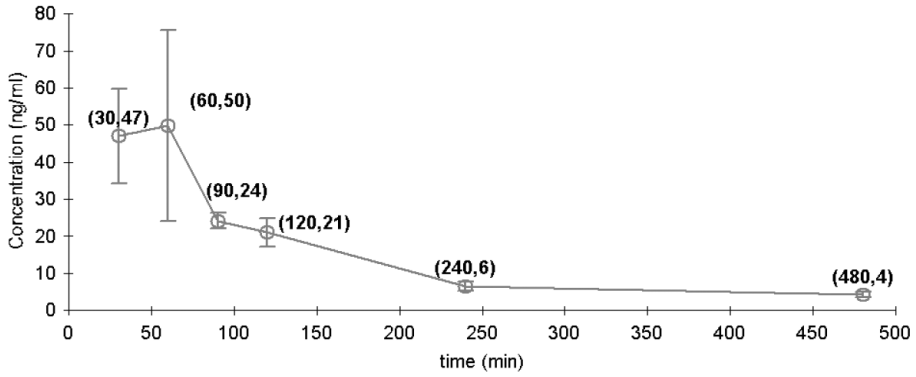

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Figure 8. Pharmacokinetics curve for AVN-211: **a**) 5 mg/kg, i.v.; **b**) 10 mg/kg, p.o.; **c**) pharmacokinetics curve, AVN-211, brain rats (10 mg/kg, p.o.)

Table 3. Main pharmacokinetic parameters of AVN-211 in rats

T_{max}	C_{max}	$AUQ_{0\rightarrow t}$	$AUC_{0\rightarrow\infty}$	$T_{1/2}$	K_{el}	Cl
min	ng/mL	ng×min/mL	ng×min/mL	min	min^{-1}	mL/min/kg
5 (i.v.)	959.7	61766.4	64921.6	50.86	0.0136	-
60 (p.o.)	204.0	29995.8	44627.8	131.15	0.0053	-
60 (p.o.) rats, brain	49.9	5589.5 ($AUQ_{0\rightarrow240}$)	7793	153.1	-	1283.2

Figure 8c shows the concentrations of AVN-211 (ng/mL) in rat brain after a single administration *per os* at the dose of 10 mg/kg. The obtained PK-plots are presented in SM. Oral bioavailability of AVN-211 was 24% in WAQ strain rats. Despite the average solubility (22 μg/mL), plasma concentrations of AVN-211 showed a linear dose-dependence up to 20 mg/kg dose given *per os*. In the 20–100 mg/kg (p.o.) range a plateau was observed. The PK features of AVN-211 were also determined in SHK mice upon intraperitoneal administration (i.p.) in doses of 1, 0.2 and 0.05 mg/kg (Table 4). The corresponding PK plots are presented in SM.

Table 4. PK parameters for AVN-211 upon i.p. administration

PK Parameters								
Dose (mg/kg)	T_{max} min	C_{max} ng/ml	$AUC_{0\rightarrow t}$ ng×min/ml	$AUC_{0\rightarrow\infty}$ ng×min/ml	$t_{1/2}$ min	K_{el} min^{-1}	V_d mL/kg	Cl mL/min/kg
1	5.0	158	3632	3726	21	0.0337	7962	268
0.2	5	67	1205	1259	23	0.0295	5381	159
0.05	5	13.70	197.18	230.59	9.88	0.0701	3091.39	216.83

One of the most crucial features of CNS-active compounds is the permeability across the blood brain barrier (BBB). For instance, among the reported bisaryl sulfonamides the following B/P ratio was reported: 0.1 (SB-271046),[59] 0.9 (indole-based analogue by Suven Life Sciences),[60] 0.19 (SB-357134),[61] 0.24 (Ro 66-0074),[62] 0.01 (Ro-04-6790 and Ro-63-0563)[59], 1.0 (4,5-dihydro-1*H*-pyrazole-containing analogue).[63] For other chemotypes including indoles, their isosteric modifications and non-sulfonyl compounds, B/P ratio is within the range of 0.5–23.[64–69] However, other PK parameters significantly influence the therapeutic efficiency of such molecules, e.g. $t_{1/2}$ (brain), Cl (brain), bioavailability, etc. Certainly, all the CNS-related PK features should be considered cumulatively to get the success. Based on the parameters above, B/P ratio for AVN-211 was calculated using different methods as following: $BrAUC_{0\rightarrow240}/PlAUC_{0\rightarrow240}$=18.6; $BrAUC_{0\rightarrow\infty}/PlAUC_{0\rightarrow\infty}$=17.5; BrC_{max}/PlC_{max}=24.4. AVN-211 was found in the rat brain after oral administration providing very good B/P ratio close to 25% based on C_{max} values. CSF/plasma ratio was also determined in rats. AVN-211 was administered *per os* in the dose of 20 mg/kg to the 6 male Wistar rats. After 60 minutes animals were anaesthetized and samples of CSF, brain and plasma were taken. CSF samples were carefully monitored for the absence of blood contaminations. AVN-211 was extracted from CSF, brain and plasma with acetonitrile and the concentrations were detected using API2000 LCMS/MS.

The obtained results are depicted in Figure 9. Summarizing, an average concentration of AVN-211 in rat CSF after oral administration in the dose of 20 mg/kg is 44.2 ng/mL, CSF/plasma ratio is 21.66%, B/P ratio is 19.5%.

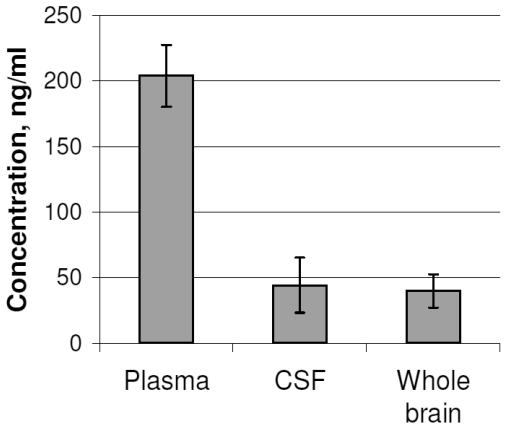


Figure 9. Relative concentrations of AVN-211 in CSF and plasma

Pharmacokinetics in Monkeys

AVN-211 (2 mg/kg, 9.4, 11.0 and 11.6 mg per animal) diluted in saline was injected into the right elbow vein in 3 mL or administered *per os* (10 mg/kg, 44 and 45 mg per animal) in 3,5 mL. No signs of an anxiety, vomiting or unusual behavior were observed. Blood was taken from the left elbow vein. The obtained results are summarized in Tables 5 and 6. The corresponding PK plots are presented in SM. Oral bioavailability of AVN-211 was calculated as a ratio of $AUC_{0\rightarrow t}$,(p.o.)/$AUC_{0\rightarrow t}$,(i.v.) and equals to 10% (F_{abs},%).

Table 5. Main PK parameters of AVN-211 upon i.v. administration in monkeys

Monkey	Route of administration	Dose (mg/kg)	PK parameters						
			T_{max}	C_{max}	C_0	$AUC_{0\rightarrow t}$	$AUC_{0\rightarrow\infty}$	$T_{1/2}$	K_{el}^*
			min	ng/mL	ng/mL	ng×min/mL	ng×min/mL	min	min^{-1}
1	i.v.	2	1	5732	7878.6	57286.3	59571.9	29.34	0.0236

2				3962	5404.9	44220.4	49402.9	43.28	0.016
3				4312	5888.3	57221.7	61673.8	35.47	0.0195
n			3	3	3	3	3	3	3
M			1	4669	6391	52909	56883	36.03	0.0197
SD			-	937	1311	7525	6563	6.99	0.0038
SEM			-	541	757	4345	3789	4.03	0.0022
CV, %			-	20.1	20.5	14.2	11.54	19.39	19.31

* K_{el} - elimination rate constant

Table 6. Main PK parameters of AVN-211 upon p.o. administration in monkeys

Monkey	**Route of administration**	**Dose (mg/kg)**	**PK parameters**					
			T_{max}	**C_{max}**	**$AUC_{0\rightarrow t}$**	**$AUC_{0\rightarrow\infty}$**	**$T_{1/2}$**	**K_{el}**
			min	ng/mL	ng×min/mL	ng×min/mL	min	min^{-1}
4	p.o.	10	15	143.0	12233	14383	212.98	0.0033
5			15	377.0	40245	41793	107.29	0.0065
n			2	2	2	2	2	2
M			15.00	260	26239	28088	160.14	0.0049
SD			-	165	19808	19381	74.73	0.0023
SEM			-	117	14006	13705	52.84	0.0016
CV, %			-	63.6	75.5	69.0	46.67	46.67

Biodistribution of the compound was examined in rats. The resulting PK curve and the main PK parameters are shown in Table 7 and Figure 10, respectively. Homogenates of liver,

heart, lungs, spleen, kidneys, brain, small and large intestines and pancreas were collected at 480 min time point and analyzed in the beta-counter. The obtained results are presented in Table 8. As clearly shown in Table 8, after oral administration at the dose of 10 mg/kg AVN-211 was found in all the studied organs. A preferential accumulation was found in large and small intestines and liver.

Table 7. PK parameters of [H^3]-AVN-211 observed in WAQ strain rats (p.o. administration)

Parameter	**Units**	**Estimate**
K_{el}	1/min	0.0006
$t_{1/2}$	min	1142
T_{max}	min	120
C_{max}	ng/mL	38.5
$AUC_{(0\rightarrow480)}$	min×ng/mL	15610
$AUC_{(0\rightarrow\infty)}$	min×ng/mL	65735
V_d/F	L/kg	251
Cl/F	(mL/min)/kg	152
MRT	min	1678

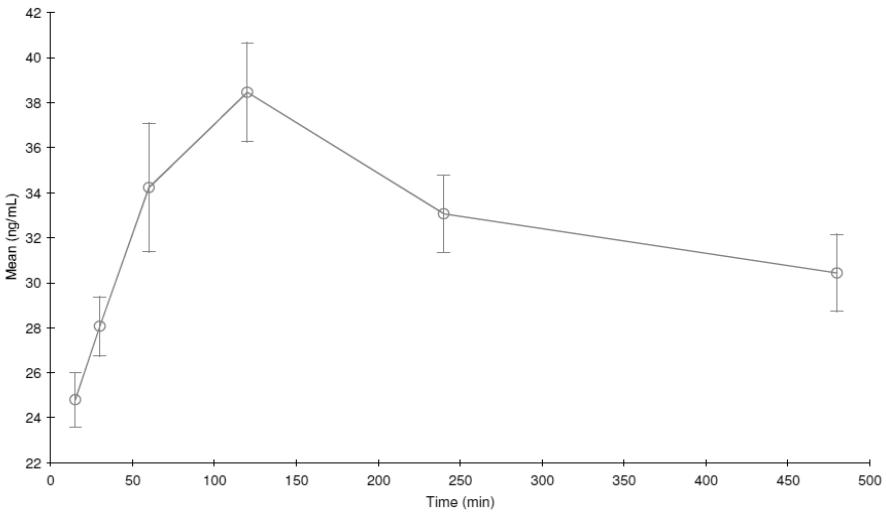


Figure 10. PK curve of AVN-211 in plasma after p.o. administration in rats

Table 8. AVN-211 concentrations in various organs and tissues

480 min	Brain	Pancreas	Large intestine	Small intestine	Liver	Lungs	Heart	Spleen	Kidneys	Blood
Concentration ng/g	13.1	31.9	91.3	66.4	57.0	33.7	20.6	13.1	41.6	32.4
	11.4	37.2	85.2	83.7	76.9	48.2	21.0	18.0	24.5	29.6
	16.3	39.4	115.7	65.7	64.5	30.8	19.0	18.6	34.5	29.3
Mean	13.6	36.2	97.4	71.9	66.1	37.6	20.2	16.6	33.5	30.4
STD	2.5	3.9	16.1	10.2	10.1	9.3	1.1	3.0	8.6	1.7

In vivo efficacy

AVN-211 was evaluated in different *in vivo* models directly associated with cognitive and memory impairments, including passive avoidance test[70,71] and Morris water maze test (MWM).[72,73] The obtained results are briefly summarized in Table 9, all the related procedures, experimental protocols and plots are presented in SM. As clearly shown in Table 9, AVN-211 can be reasonably regarded as novel promising drug candidate for the treatment of AD as well as

schizophrenia. In many cases, the observed effects were considerably higher than that demonstrated for PRX-07034 and SB-742457 or comparative to Donepezil, Memantine, Buspirone, Lorazepam, Fenobam, Rufinamide, Piracetam, Haloperidol, and Apomorphine.

Table 9. *In vivo* efficacy of AVN-211*

Description	Study	Subject	Brief summary
Scopolamine-Based Models of Amnesia	**Passive Avoidance Test**: scopolamine was administered i.p. at the dose of 0.3 mg/kg, 30 min prior training	BALB/c mice (24–25 g)	AVN-211 (0.05 - 1 mg/kg, i.p. or 0.2 mg/kg, p.o.) was more effective than Memantine (5 mg/kg, i.p.) or Tacrine (10 mg/kg, i.p.), while PRX-07034 (10 mg/kg, i.p.) was not active in the test. SB-742457 (1 mg/kg, i.p.) was slightly active affecting time spent in the light chamber of the passive avoidance apparatus. The most pronounced effect of AVN-211 was observed at 0.05 mg/kg (i.p.) and 0.2 mg/kg (p.o.) doses
	Passive Avoidance Test: scopolamine (2.5 mg/kg, i.p.) 20 min before the test	10-wk old male and female Wistar rats (160–185 g)	Given either *per os* or i.p. AVN-211 (0.2-5 mg/kg, 30 min before the test) significantly restored memory in scopolamine-treated rats. The control group animals were injected with physiological solution
	Morris Water Maze Test: (*a*) scopolamine (1.5 mg/kg, i.p.) or (*b*) scopolamine (1.5 mg/kg,	BALB/c mice (24–25 g)	Scopolamine was administered 30 min prior training, Tacrine and Donepezil – 60 min before training, while AVN-211 - 5 min prior training. The control group animals

	i.p.) combined with Tacrine (3 mg/kg, i.p.), Donepezil (3 mg/kg, i.p.) or AVN-211 (0.05, 0.2 or 1 mg/kg, i.p.)		were injected with physiological solution. AVN-211 showed antiamnestic activity comparable to that of AChE inhibitor Donepezil (Aricept)[74]
	Morris Water Maze Test: (a) scopolamine (4.5 mg/kg, i.p.) or (*b*) scopolamine (4.5 mg/kg, i.p.) combined with AVN-211 (0.2 mg/kg, i.p.)	Wistar rats (160–185 g, 13–14 wk old)	Scopolamine was administered 30 min before training, while AVN-211 - 5 min prior training. AVN-211 was active effectively restoring time in target zone almost to a placebo level. The control group animals were injected with physiological solution
	Novel Object Recognition Test: the test was abolished by scopolamine administered in the dose of 1 mg/kg i.p. 30 minutes before the training.	SHK mice (24–30 g)	The scopolamine-induced deficit in novel object recognition was prevented by administration of Memantine (10 mg/kg, i.p.) 60 min prior the training, AVN-211 (0.05 and 0.2 mg/kg, i.p.) 5 minutes before the training, or SB-742457 (0.2 mg/kg, i.p.) 15 min before the training but not PRX-07034 (10 mg/kg, i.p.), 15 minutes before the test. AVN-211 showed an anti-amnesic effect exceeding that of SB-742457 and Memantine
MK-801- Based Models of Amnesia	**Passive Avoidance Test**: on day of training, mice were treated with pro-amnesic	BALB/c mice (24–25 g)	Independent groups of mice were treated additionally with one of the following drugs: Tacrine, 10 mg/kg, i.p., 60 min

	agent MK-801 (0.1 mg/kg, i.p.) 30 minutes prior the training. MK-801 pre-treatment significantly reduced effect of the training producing so-called anterograde amnesia		before the training, Memantine, 10 mg/kg, i.p., 60 min prior the training, SB-742457, 1 mg/kg, i.p., 15 min before the training, PRX-07034, 10 mg/kg, i.p., 30 min before the training, or with AVN-211 (0.02-0.2 mg/kg, i.p.), which was administered 5 min before the test. The control group animals were injected with physiological solution. AVN-211 was more effective compared to SB-742457, while Memantine, Tacrine and PRX-07034 were not active in the test
	Morris Water Maze Test: mice were treated with (*a*) MK-801 (0.2 mg/kg, i.p.) or (*b*) MK-801 (0.2 mg/kg, i.p.) combined with Tacrine (3 mg/kg, i.p.), Donepezil (1 mg/kg, i.p.), Memantine (5 mg/kg, i.p.), or AVN-211 (0.05 or 0.2 mg/kg, i.p.). MK-801 was administered 30 min prior the test. Tacrine, Donepezil and Memantine were administered 60 min before the training	BALB/c mice (24–25 g)	AVN-211 was administered 5 min prior to the training. The control group animals demonstrated successful learning, since they spent more time in the quadrant of the platform location on the 3rd day. MK-801 at the dose of 0.2 mg/kg disrupted training process under the described conditions. At the same time, co-administration of Memantine or AVN-211 (0.05 mg/kg) significantly improved the learning phase of spatial navigation

	Morris Water Maze Test: the experiment was performed as described above with only one modification – MK-801 (0.2 mg/kg, i.p.) was used instead of scopolamine. MK-801 was injected 30 min before the training	male and female Wistar rats (160–185 g, 10 wk old)	AVN-211 (0.2 mg/kg, i.p.) was active in the test effectively restoring time in target zone to a placebo level
	Novel Object Recognition Test: MK-801 was administered in the dose of 0.2 mg/kg i.p. 30 minutes before the training. The MK-801-induced deficit in novel object recognition was not prevented by co-administration of Tacrine (3 mg/kg, i.p.) 60 min prior the training or AVN-211 (0.05-1 mg/kg, i.p.) 5 minutes before the training	SHK mice (22–32 g)	AVN-211 as well as all the tested reference molecules did not reveal an antiamnesic effect within the test under the applied conditions
Anxiolytic Activity	**Elevated Plus Maze**: mice were treated with either placebo, or Buspirone (5 mg/kg, i.p., 30 min prior the	BALB/c mice (24 g)	Buspirone and Lorazepam were administered at the maximal dose which did not produce sedative side-effects seen in the test as a decrease in a general exploratory

	training), or Lorazepam (0.05 mg/kg, i.p., 60 min before the training), or Fenobam (5 mg/kg, i.p., 60 min before the training), or Rufinamide (15 mg/kg, i.p., 60 min prior the test) or with AVN-211 (0.05, 0.2, or 1 mg/kg, i.p., 5 min before the training)		activity. Buspirone, Lorazepam, Fenobam, Rufinamide and AVN-211 produced a clear anxiolytic effect in the elevated plus maze test: they significantly increased the number of visits into the open arms of the maze, time spent in the open arms and decreased the number of defecations. AVN-211, Lorazepam, Buspirone, Fenobam and Rufinamide did not affect locomotor activity, thus their anxiolytic activity was not related to a sedative effect. The most prominent anxiolytic effect was seen with AVN-211 injected i.p. at the doses of 0.01–0.2 mg/kg, Lorazepam injected i.p. at the dose of 0.05 mg/kg and Fenobam injected i.p. in the dose of 5 mg/kg
	Elevated Platform Test	Male Sprague-Dawley rats (250–300 g)	Both lorazepam (0.05 mg/kg, i.p.), 60 minutes before the test, and AVN-211 (0.05 mg/kg, i.p.), 5 minutes before the test, decreased the total time of freezing during the first minutes in the test
	Open Field Test: Lorazepam (0.05 mg/kg, i.p.) was administered 30 minutes prior the testing and AVN-211 (0.05 and 0.2 mg/kg, i.p.) was	SHK mice (20–25 g)	AVN-211 and Lorazepam increased the number of entries and the total time spent in the central zone

	administered 5 minutes prior to the testing		
Antipsychotic Activity	**Pre-pulse Inhibition of Acoustic Startle**: apomorphine was dissolved in 0.1% ascorbic acid in sterilized water. Haloperidol was dissolved in Tween 80. AVN-211 was dissolved in sterile water with 0.1% Tween 80. The appropriate vehicle solution was sterile water with 0.1% Tween 80. Haloperidol was administered 60 min prior to the testing (volume of injection was 10 ml/kg). Apomorphine was administered s.c. 20 min before the testing (volume of injection was 1 ml/kg.). AVN-211 was administered i.p. 5 min before the testing (volume of injection was 10 ml/kg)	Naive male SHK (24–30 g),	The obtained results demonstrated about 53% pre-pulse inhibition in the placebo group. Pro-psychotic agent apomorphine (3 mg/kg) reduced this variable showing deterioration of ability for filtration of sensory signals. AVN-211 (0.05 and 0.2 mg/kg) and Haloperidol (1 mg/kg) prevented disruptive effect of apomorphine on the startle pre-pulse inhibition. AVN-211 at doses of 0.05 and 0.2 mg/kg revealed anti-psychotic-like properties. Prepulse inhibition (% to pulse alone) for apomorphine+placebo was 29%, for Haloperidol+apomorphine – 55%, for AVN-211+apomorphine – 46% (0.05 mg/kg) and 42% (0.2 mg/kg)
Nootropic	**Cognitive Deficit in**	SHK Mice	The test revealed that AVN-211 and

Activity	**Exploratory Low**: on days 4-7, the mice o.d. received i.p. injection of placebo, piracetam (400 mg/kg, i.p.) or AVN-211 (0.1 mg/kg, i.p.). The second cross-maze test was performed on Day 7 an hour after the last injection	(22–32 g)	piracetam produce a marked decrease in number of visits during the 1st patrolling demonstrating a clear cognition-enhancing effect. Number of arm visits: placebo (EH mice) – av. 5.5, placebo (EL mice) – av. 5.7, Piracetam (400 mg/kg, EL mice) – av. 5.1, AVN-211 (0.1 mg/kg, EL mice) – av. 5.6. EH (exploratory high) or EL (exploratory low) animal subgroups

* - ref. compounds were evaluated in doses published previously in scientific literature. In some cases, dose was dependent on exp. conditions and trial specificity. AVN-211, SB-742457 and PRX-07034 were evaluated in different doses (*titrated*).

Safety pharmacology and side-effects

*h*ERG channel inhibition

Drugs belonging to different classes have been shown to be associated with QT prolongation leading in some cases to serious ventricular arrhythmias. The most common mechanism for these adverse events is the inhibition of one or more cardiac potassium channels, in particular *h*ERG channel. Potassium currents controlled by this channel are important for re-polarization of cardiac myocytes and unintended blockage of these channels by some drugs causes prolonged myocardial QT interval with potentially lethal consequences. AVN-211 was tested for its ability to block *h*ERG channel using HEK cells stably expressing the *h*ERG potassium channel (*for details, see SM*). Thus, AVN-211 exhibited a potency to block *h*ERG channel two orders of magnitude lower than that of blocking serotonin-induced cell response mediated through 5-HT_6R (Figure 4). In the patch clamp experiments (ion current inhibition) the compound showed an IC_{50} value of 18.0 μM. Taking into account such a low potency, we expect that AVN-211 will not affect QT interval *in vivo*.

Assessment of LD_{50}

AVN-211 was injected in mice i.p. 10 ml/kg in different doses ranged from 500 to 20000 mg/kg (Figure 11). As shown in Figure 11, AVN-211 should be assigned to non-toxic organic compounds and demonstrates the LD_{50} value of 13684±2370 mg/kg.

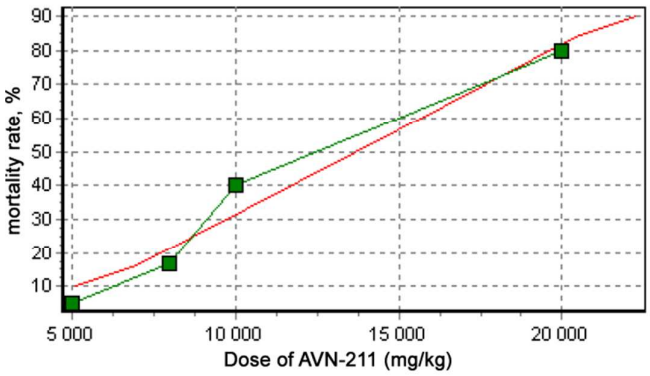


Figure 11. Mortality among SHK mice upon AVN-211 treatment in different doses

AVN-211 was injected i.p. daily during 5 days in 10, 30, 100, 200 and 400 mg/kg doses (only 200 and 400 mg/kg groups data presented). Each experimental group consisted of 8 mice. Animal weight and mortality were measured. Injection of AVN-211 did not cause statistically significant reduction in body weight in comparison to the control group (Figure 12). The data suggests that MTD of AVN-211 is not less than 400 mg/kg.

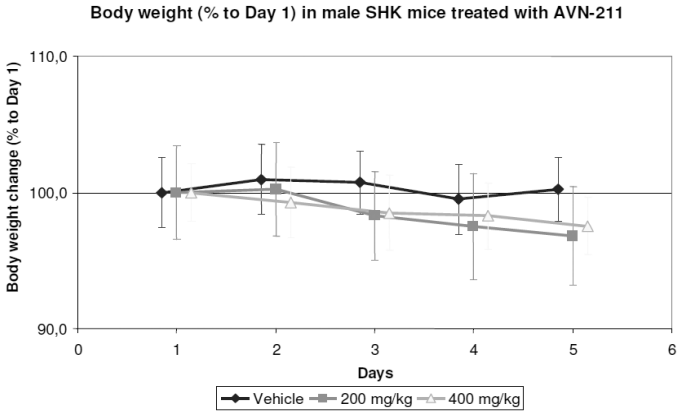


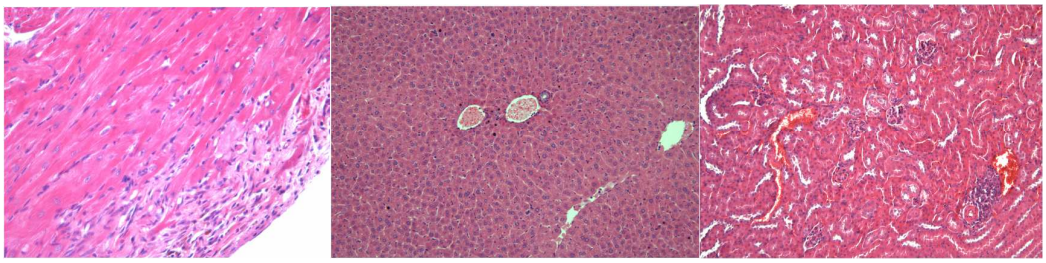
Figure 12. Determination of MTD for AVN-211

14-Days toxicity study was performed in 20 male adult SHK mice (20-25 g). During the trial no statistically significant change in the enzymatic activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as in the level of glucose and urea in plasma was observed after 14-days treatment with AVN-211 (i.p., o.d. 20 mg/kg), however a trend in a body weight decrease was detected and might be attributed to a mechanism-based action as described above.

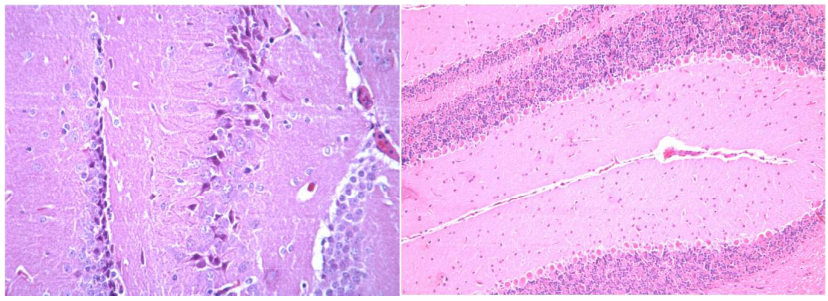
Morphology of internal organs was examined visually and microscopically (paraffin slices 4–5 μm, hematoxylin-eosin staining). Mass coefficients of internal organs were determined by weighing respective organs: thymus, heart, lungs, kidneys, spleen, brain and cerebellum. Necropsy of the mice was performed after 14 days of AVN-211 i.p. administration in the dose of 20 mg/kg. Five males and five females were studied. Animals were sacrificed by decapitation. Study was performed within an hour after decapitation. Samples of heart, liver, kidneys, brain and cerebellum were collected for histological analysis. Clinical observation revealed no signs of toxicity: no hair loss, glossy and shiny hair, skin is elastic, sub-epidermal cellular tissue moderately expressed (Figure 13, Table 10).

Appearance and condition of internal organs in control and AVN-211-treated mice were similar (Table 10): lungs – lobes correctly shaped, pale pink colored, foamy liquid is seen on the slice; *liver* – regular size, trabecules (no enlargement), dark brown, plethoric, intralobular capillary network was developed, hepatocytes and Kupfer cells were regularly shaped; *spleen* – elongated, purple, moderately solid; *kidneys* – light brown, fibrous capsule was shiny and thin, easily removable, capillaries of glomerules of cortical substance were plethoric, no alterations in epithelium of proximal and distal tubules, tubular glimpses were open; *brain* - regular size, brain tunics were uniformly colored without visible hemorrhages, cortex and subcortical formations were of normal shape and appearance, ventricles of brain were of a normal size (not enlarged); *cerebellum* – regular size, no alterations in composition, no visible hemorrhages, all structural elements were without alterations; *heart* – was seen on the slices as longitudinal and transverse

muscle fibers, coronary vessels of a regular appearance; *Purkinje cells* were regularly shaped, blood vessels were not enlarged, study of mass coefficient of internal organs did not reveal statistically significant difference between control and AVN-211-treated animals. Organs of both groups (control and AVN-211 20 mg/kg i.p.) were observed using microscope and located regularly. Based on the obtained results, it can be concluded that AVN-211 did not show any signs of toxicity during the study performed. Similar results were obtained in rats (see SM).



a **b** **c**



d **e**

Figure 13. AVN-211 treated mice: (**a**) cardiac wall, endocardium, no alterations observed (hematoxylin, eosin 40x10); (**b**) liver, no alterations, hematoxylin and eosin, 20x10; (**c**) kidneys, no alterations, hematoxylin and eosin, 20x10; (**d**) hippocampal formation, pyramidal neurons and granular cells without alterations (hematoxylin and eosin, 40x10); (**e**) cerebellum, structural elements and Purkinje cells without alterations (hematoxylin, eosin, 20x10).

Table 10. Mass coefficients of internal organs after 14 days of the study

Group	**Organ mass coefficient (g/kg body weight)**

	Thymus	Lungs	Heart	Liver	Spleen	Kidneys	Brain	Cerebellum
Control	2.8±0.2	8.9±0.4	5.2±0.1	59.4±3.6	6.3±1.3	15.4±0.6	9.7±0.3	3.8±0.1
AVN-211 20 mg/kg	3.0±0.3	9.5±0.6	5.5±0.3	52.0±1.7	5.9±1.0	15.4±0.9	10.5±0.5	3.6±0.3

Morphology of internal organs, body weight dynamics, general behavior (open field test and grid test), plasma biochemistry as well as hematology were evaluated by analogy to aforementioned approaches during the 29- and 30-days trial. During the first study, the compound was tested in doses of 1, 3 and 10 mg/kg and no toxicity was revealed. This observation was supported by lack of a body weight change in all animal groups. General behavior tests did not reveal any signs of toxicity. There were no changes related to AVN-211 administration in micro-structure of studied organs, while some changes in mass coefficient (liver, lungs, testicles) were not related to AVN-211 administration.

In the second trial, AVN-211 administered in the dose of 3 mg/kg orally was not toxic as well. No changes in the body weight, parameters of plasma biochemistry markers, macro- and microscopic observations were detected. Observed alterations in the lung parenchyma could be a consequence of distress syndrome. It should be noted that the estimation of 30-days toxicity of AVN-211 (10 mL/kg, p.o.) in monkeys revealed no toxicity except minor alterations in blood plasma biochemistry that could be explained by ketamine tranquilization.

The signs of adverse effects were revealed during the long-term 180-days toxicological study. Thus, statistically significant body weight reduction was observed in male and female rats in 1 mg/kg group. This can be explained either by moderate toxicity caused by AVN-211 administration or by AVN-211 action as a 5-HT_6R antagonist. However, no dose dependent weight reduction was found in AVN-211-treated female rats suggesting rather 5-HT_6R-related than toxicological explanation of body weight reduction in female rats. With respect to plasma biochemistry, the increase in bilirubin level was observed at the highest dose in males which can

be associated with liver damage. However, such outcome was not observed in pathomorphological experiments. Macroscopic study of the organs did not reveal any difference between placebo and AVN-211-treated animals. Thus, as a result of conducted pathomorphological investigation, no toxic action of AVN-211 administered orally during 180 days was observed (. The noted special features and changes in the histological structure of the internal organs of experimental animals did not bear the signs of pathology, they did not have noticeable dose dependence and, often, they were manifested by similar means in animals of placebo group as well.

AVN-211 in concentrations up to 200 μM was not mutagenic according to the results obtained in Ames test. Results of two independent experiments support this conclusion. No significant chromosomal aberrations were found in AVN-211-treated mice, suggesting absence of AVN-211-induced mutagenic activity *in vivo*. According to F-LIGRLO-induced paw edema test, AVN-211 did not reveal any allergenic properties in the doses of 1, 3 and 10 mg/kg.

We also demonstrated that scopolamine, in gastrointestinal transit test performed in mice, produced marked increase in time of first defecation after charcoal administration showing decrease in GI transit velocity, whereas AVN-211 did not produce any significant effect on the transit.

Catalepsy was evaluated in rats by method adopted from studies by Bourson[75] and Bristow.[76] It was revealed that AVN-211 did not produce cataleptic effect in either of the tested doses (0.2 and 1 mg/kg) while haloperidol caused a pronounced cataleptic effect.

Considering the aforementioned results, AVN-211 can be reasonably regarded as generally non-toxic organic substance with promising ADME features and activity for further clinical evaluation against AD.

DISCUSSION

The results above clearly show that AVN-211 leads to considerable improvements in cognition and learning investigated in different behavioral models of AD. *In vivo* efficacy of AVN-211 was compared to that of Memantine (a fixed-dose combination of memantine hydrochloride and donepezil hydrochloride was launched in 2015, U.S., by Actavis against AD-type dementia), Tacrine (launched in 1993 by Shionogi), SB-742457 (Phase II, GSK) and PRX-07034 (Phase II, Epix Pharmaceuticals). During all the behavioral tests performed, AVN-211 significantly restored both scopolamine- and MK-801-induced cognitive dysfunction. In some cases, the cognition restorative effect of the lead molecule was higher as compared with that of the control compounds. Additionally, in an elevated plus-maze model, elevated platform and open field tests AVN-211 has shown a similar or better anxiolytic efficacy than fenobam, rufinamide, lorazepam and buspirone. Additionally, AVN-211 has a clear IP and easily scalable chemistry, allowing us to consider the molecule as a new promising drug candidate for the treatment of neurodegenerative and psychiatric disorders.

Among the current trends matured in AD drug treatment the re-profiling of selective 5-HT_6R ligands, especially antagonists, can be highlighted. Thus, Lu AE58054 was evaluated in Phase II clinical trial in Lundbeck against schizophrenia. However, in 2010, in spite of some initial success,[77] the company discontinued the development of the compound for the treatment of cognitive impairment associated with schizophrenia (CIAS) based on efficacy data which did not support further development for this indication.[78] Intriguingly, Lundbeck has recently initiated the extensive clinical trials of Lu AE58054 as adjuvant therapy against AD, including four Phase III,[79,80–82] since Phase II evaluation was successfully completed in 2012.[83–85] In 2013, the compound was licensed by Lundbeck to Otsuka Pharmaceutical for co-development and co-commercialization in Canada, the U.S., certain East Asian countries, including Japan, major European countries and Nordic countries for the treatment of AD-type dementia.

SB-742457, developed by GSK, was initially focused against schizophrenia, depression, anxiety and AD.[85–90] The company successfully completed preclinical evaluation of the

compound mainly focusing on common cognitive disorders then made the decision to advance this molecule in clinics exclusively against AD. As a result, Phase II clinical trial was completed demonstrating that SB-742457 was generally safe and well tolerated and may be efficacious in AD.[91] In last quarter of 2014, Roivant Neurosciences has entered into an agreement with GSK for the acquisition of SB-742457. GSK conducted 13 clinical studies of an excellent safety and a some involving over 1,250 healthy subjects and 357 patients with AD. The results of these studies provide some evidence that SB-742457 holds promise for patients with mild to moderate AD, however, no relevant proofs were obtained following this indication.[92]

Roivant intends to meet with the FDA in the first half of 2015 to confirm the regulatory requirements for the continued development of the product for adjuvant therapy of patients with mild to moderate AD. Phase III clinical evaluation has recently been launched for RVT-101 (re-named SB-742457) against AD.[93] The company may also explore the clinical development of SB-742457 for other neurological disorders for which there are limited or no therapeutic options.

Avineuro Pharmaceuticals, Inc. has conducted Phase I and Phase IIa clinical trials with AVN-211 as adjuvant therapy in patients with schizophrenia in a condition of partial remission receiving stable antipsychotic therapy. The results of this a multicenter double-blind randomized placebo-controlled study supported good safety and tolerability profiles of AVN-211, as well as gave an encouraging perspective in this adjuvant therapy. We plan to continue clinical trials AVN-211 as adjuvant therapy in patients with schizophrenia in partial remission.[94,95]

Summarizing the results above, selective 5-HT_6R antagonists have recently emerged as a prominent class of small-molecule compounds with an enormous therapeutic potential against AD. The comprehensive preclinical evaluation presented here allows us to make a decision towards the initiation of a separate clinical program against AD, since AVN-211 showed high efficiency and low toxicity in a range of *in vivo* animal tests specific for this neuropathology. Clinical advances of Lu AE58054 and SB-742457 in the adjuvant therapy of AD obtained in recent years firmly confirm the validity of the approach.[95] However, despite a range of great

achievements, several preclinical studies have indicated rather controversial results obtained for the reported 5-HT_6R antagonists.[96] For instance, the beneficial effects of 5-HT_6R antagonists disclosed in early animal studies during the MWM test could not be replicated.[97-99] Moreover, SB-271046 demonstrated a relatively poor or not detectable efficiency at all towards the scopolamine-induced learning deficit in the MWM test[100] and contextual fear conditioning (CFC) paradigms.[98] The compound also did not show clozapine-like effects in models for positive and negative symptoms of schizophrenia.[101] In addition, during a typical object recognition test Ro-4368554 had no impact on the delay-induced recognition deficit.[102] SB-258585 (3–30 mg/kg, i.p.) and Ro-4368554 (1–10 mg/kg, i.p.) have recently been tested in different models for schizophrenia, including apomorphine-induced deficit in PPI of acoustic startle and amphetamine-evoked locomotor activity in rats. In addition, the therapeutic effects of both compounds on learning and memory deficits in rats produced by natural forgetting and by MK-801 or scopolamine, in the contextual fear conditioning, MWM and spontaneous alternation paradigms has assessed.[96] As a results, it was revealed that Ro-4368554 increased activity at 1 mg/kg but not at higher doses, whereas SB-258585 had no effect on the spontaneous locomotor activity. Ro-4368554 and SB-258585 enhanced memory, as evidenced by an increase in the exploration of the new object versus the old object. Ro-04-6790 and SB-271046 prevented scopolamine-induced amnesia in the PA test.[103,104] However, in a model of cholinergic dysfunction, that is, scopolamine-induced memory impairment (AD-related model), Ro-4368554 and SB-258585 were unable to prevent the impairment induced by scopolamine in CFC and passive avoidance tests, as it was demonstrated previously for Ro 04-6790 and SB-271046.[102] In MWM model, SB-258585 showed a beneficial effect on memory in contrast to Ro-4368554.[96,105] Neither Ro-4368554 or SB-258585 reversed the effect of amphetamine in amphetamine-induced hyperlocomotion in the open field and apomorphine-induced impairment of PPI (two models for psychotic-like symptoms).[96] However, SB-258585 significantly enhanced amphetamine-induced hyperlocomotion at the highest tested dose of 30 mg/kg, while Ro-4368554 was able to reverse

apomorphine-induced deficits in PPI at a low prepulse intensity. The analogues comparative *in vivo* tests have been performed for AVN-211 and the corresponding results are summarized in Table 9 (*vide supra*) indicating the enhanced therapeutic effect in contrast to the reference compounds. Therefore, good clinical perspectives can be imagined for AVN-211 keeping in mind the upper selectivity and efficiency over the reported drug candidates revealed during an extensive preclinical study disclosed herein. Indeed, a substantial enhancement of both chemically-impaired and non-impaired cognition in mice and rats given at low doses makes AD as a primary therapeutic indication for AVN-211. Additionally, AVN-211 has shown a considerable anxiolytic effect that allows us to consider the molecule for alternative therapeutic indication. This compound has the best selectivity profile among the reported 5-HT_6R antagonists and appropriate ADME features reliable for oral administration. The results of subsequent clinical evaluation of AVN-211 against AD will be disclosed in a follow up communication.

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SUPPORTING INFORMATION

The supplementary material is available in the electronic version of this article.

REFERENCES

(1) World Health Organization, http://www.who.int/en, accessed 16 July, 2015.

(2) Zawieja, P.; Kornprobst, J.M.; Métais, P. 3-(2,4-dimethoxybenzylidene)-anabaseine: a promising candidate drug for Alzheimer's disease? *Geriatr Gerontol Int.* **2012**, *12* (3), 365–371.

(3) Weinreb, O.; Amit, T.; Bar-Am, O.; Youdim, M.B. Ladostigil: a novel multimodal neuroprotective drug with cholinesterase and brain-selective monoamine oxidase inhibitory activities for Alzheimer's disease treatment. *Curr Drug Targets* **2012**, *13* (4), 483–494.

(4) A PET Study of the Effects of p38 MAP Kinase Inhibitor, VX-745, on Amyloid Plaque Load in Alzheimer's Disease (AD). NCT02423122, https://clinicaltrials.gov/ct2/show/NCT02423122, accessed 16 July 2015.

(5) Fukunaga, K.; Yabuki, Y. Alzheimer therapeutics ST101 improves the cognition in schizophrenia model rats. *11th World Congr Biol Psychiatry* **2013**, Abst 024.

(6) Khanferyan, R.; Chekanova, O.; Dorofeeva, J.; Galenko-Jaroshevsky, A. WoS Record Antihistamine drug, Dimebon, with multiple mechanisms of actions. *Inflamm Res.* **2011**, *60*, S336.

(7) Schneider, L.S. Idalopirdine for Alzheimer's disease: written in the stars. *Lancet Neurol.* **2014**, *13* (11), 1063–1065.

(8) Marazziti, D.; Rutigliano, G.; Catena-Dell'Osso, M.; Baroni, S.; Dell'Osso, L. The 5-HT_6 receptor antagonism approach in Alzheimer's disease. *Drugs Fut.* **2014**, *39* (2), 133.

(9) Maher-Edwards, G.; Zvartau-Hind, M.; Hunter, A.J.; Gold, M.; Hopton, G.; Jacobs, G.; Davy, M.; Williams, P. Double-blind, controlled phase II study of a 5-HT_6 receptor antagonist, SB-742457, in Alzheimer's disease. *Curr Alzheimer Res.* **2010**, *7* (5), 374–385.

(10) Moebius, H.; Loewen, G.; Dgetluck, N.; Hilt, D. A randomized, double-blind, placebo-controlled, 24-week, phase 2b outcomes study of 3 different doses of encenicline or placebo

in subjects with mild to moderate probable Alzheimer's disease. *67th Annu Meet Am Acad Neurol (AAN)* **2015**, Abst P7.100.

(11) AC-1204 26-week long term efficacy Response trial with optional open-label ext (NOURISH AD). NCT01741194, https://clinicaltrials.gov/ct2/show/NCT01741194, accessed 16 July 2015.

(12) Scott, J.D.; Li, S.W.; Cartwright, M. Discovery of MK-8931: A BACE inhibitor in phase 3 clinical development for Alzheimer's disease. *249th Am Chem Soc (ACS) Natl Meet.* **2015**, Abst MEDI 246.

(13) Alexander, R.; Haeberlein, S.B.; Rosen, L. AZD3293 a novel BACE1 inhibitor: Pharmacokinetics and effects on plasma and CSF A-beta peptides following multiple-dose administration in Alzheimer's disease patients. *12th Int Conf Alzheimer Parkinson Dis (AD/PD)* **2015**, Abst 091.

(14) Baddeley, T.C.; McCaffrey, J.; Storey, J.M.; Cheung, J.K.; Melis, V.; Horsley, D.; Harrington, C.R.; Wischik, C.M. Complex disposition of methylthioninium redox forms determines efficacy in tau aggregation inhibitor therapy for Alzheimer's disease. *J Pharmacol Exp Ther.* **2014**, *352* (1), 110–118.

(15) Lermontova, N.N.; Redkozubov, A.E.; Shevtsova, E.F.; Serkova, T.P.; Kireeva, E.G.; Bachurin, S.O. Dimebon and tacrine inhibit neurotoxic action of beta-amyloid in culture and block L-type Ca^{2+} channels. *Bull Exp Biol Med.* **2001**, *132*, 1079–1083.

(16) Bachurin, S.O.; Shevtsova, E.P.; Kireeva, E.G.; Oxenkrug, G.F.; Sablin, S.O. Mitochondria as a target for neurotoxins and neuroprotective agents. *Ann N Y Acad Sci.* **2003**, *993*, 334–344.

(17) Grigorev, V.V.; Dranyi, O.A.; Bachurin, S.O. Comparative study of action mechanisms of dimebon and memantine on AMPA- and NMDA-subtypes glutamate receptors in rat cerebral neurons. *Bull Exp Biol Med.* **2003**, *136*, 474–477.

(18) Cano-Cuenca, N.; Sol´ıs-Garc´ıa del Pozo, J.E.; Jorda´n, J. Evidence for the Efficacy of Latrepirdine (Dimebon) Treatment for Improvement of Cognitive Function: A Meta-Analysis. *Journal of Alzheimer's Disease* **2014**, *38*, 155–164.

(19) Doody, R.S.; Gavrilova, S.I.; Sano, M.; Thomas, R.G.; Aisen, P.S.; Bachurin, S.O.; Seely, L.; Hung, D. Effect of dimebon on cognition, activities of daily living, behaviour, and global function in patients with mild-to-moderate Alzheimer's disease: A randomised, double-blind, placebo-controlled study. *Lancet* **2008**, *372*, 207–215.

(20) Negative Phase III trial results for Dimebon. *Alzheimer's Association*, http://www.alz.org/norcal/in_my_community_19452.asp, accessed 16 July 2015.

(21) Dimebon disappoints in Phase III trial of Huntington's Disease. *Medscape*, http://www.medscape.com/viewarticle/740692, accessed 16 July 2015.

(22) Dar, T.A.; Sheikh, I.A.; Ganie, S.A.; Ali, R.; Singh, L.R.; Gan, S.H.; Kamal, M.A.; Zargar, M.A. Molecular linkages between diabetes and Alzheimer's disease: current scenario and future prospects. *CNS Neurol Disord Drug Targets* **2014**, *13* (2), 290–298.

(23) Talbot, K.; Wang, H.Y. The nature, significance, and glucagon-like peptide-1 analog treatment of brain insulin resistance in Alzheimer's disease. *Alzheimers Dement.* **2014**, *10* (1Suppl), S12–25.

(24) Wang, Y.; Serricchio, M.; Jauregui, M.; Shanbhag, R.; Stoltz, T.; Di Paolo, C.T.; Kim, P.K.; McQuibban, G.A. Deubiquitinating enzymes regulate PARK2-mediated mitophagy. *Autophagy* ***2015***, *11* (4), 595–606.

(25) Perluigi, M.; Di Domenico, F.; Butterfield, D.A. mTOR signaling in aging and neurodegeneration: At the crossroad between metabolism dysfunction and impairment of autophagy. *Neurobiol Dis.* **2015** doi: 10.1016/j.nbd.2015.03.014.

(26) Landreth, G.E.; Reed-Geaghan, E.G. Toll-like receptors in Alzheimer's disease. *Curr Top Microbiol Immunol.* **2009**, *336*, 137–153.

(27) Cho, S.J.; Yun, S.M.; Jo, C.; Lee, D.H.; Choi, K.J.; Song, J.C.; Park, S.I.; Kim, Y.J.; Koh, Y.H. SUMO1 promotes Aβ production via the modulation of autophagy. *Autophagy* **2015**, *11* (1), 100–112.

(28) Skaper, S.D. Wnt-signalling: A new direction for alzheimer disease? *CNS Neurol Disord Drug Targets* **2014**, *13* (4), 556.

(29) Shibuya, Y.; Niu, Z.; Bryleva, E.Y.; Harris, B.T.; Murphy, S.R.; Kheirollah, A.; Bowen, Z.D.; Chang, C.C.; Chang, T.Y. Acyl-coenzyme A:cholesterol acyltransferase 1 blockage enhances autophagy in the neurons of triple transgenic Alzheimer's disease mouse and reduces human P301L-tau content at the presymptomatic stage. *Neurobiol Aging* **2015**, *36* (7), 2248–2259.

(30) Tanzi, R.E. TREM2 and Risk of Alzheimer's Disease - Friend or Foe? *N Engl J Med.* **2015**, *372* (26), 2564–2565.

(31) Yan, R.; Vassar, R. Targeting the β secretase BACE1 for Alzheimer's disease therapy *Lancet Neurol.* **2014**, *3* (3), 319–329.

(32) Hall, A.; Patel, T.R. γ-Secretase modulators: current status and future directions. *Prog Med Chem.* **2014**, *53*, 101–145.

(33) de Bruin, N.M.; Kruse, C.G. 5-HT_6 Receptor Antagonists: Potential Efficacy for the Treatment of Cognitive Impairment in Schizophrenia. *Curr Pharm Des.* 5 Jun 5 (published online).

(34) Benhamú, B.; Martín-Fontecha, M.; Vázquez-Villa, H.; Pardo, L.; López-Rodríguez, M.L. Serotonin 5-HT_6 receptor antagonists for the treatment of cognitive deficiency in Alzheimer's disease. *J Med Chem.* **2014**, *57* (17), 7160-7181.

(35) Meffre, J.; Chaumont-Dubel, S.; Mannoury la Cour, C.; Loiseau, F.; Watson, D.J.; Dekeyne, A.; Séveno, M.; Rivet, J.M.; Gaven, F.; Déléris, P.; Hervé, D.; Fone, K.C.; Bockaert, J.; Millan, M.J.; Marin, P. 5-HT_6 receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia. *EMBO Mol Med.* **2012**, *4* (10), 1043–1056.

(36) Marin, P.; Meffre, J.; Bockaert, J. The mTOR pathway: a new target for treating cognitive deficits of schizophrenia? *Med Sci (Paris)* **2013**, *29* (1), 28–30.

(37) Geldenhuys, W.J.; Van der Schyf, C.J. The serotonin 5-HT_6 receptor: a viable drug target for treating cognitive deficits in Alzheimer's disease. *Expert Rev Neurother*. **2009**, *9* (7), 1073–1085.

(38) Ramirez, M.J.; Lai, M.K.; Tordera, R.M.; Francis, P.T. Serotonergic therapies for cognitive symptoms in Alzheimer's disease: rationale and current status. *Drugs* **2014**, *74* (7), 729–736.

(39) Geldenhuys, W.J.; Van der Schyf, C.J. Serotonin 5-HT_6 receptor antagonists for the treatment of Alzheimer's disease. *Curr Top Med Chem*. **2008**, *8* (12), 1035–1048.

(40) Upton, N.; Chuang, T.T.; Hunter, A.J.; Virley, D.J. 5-HT_6 receptor antagonists as novel cognitive enhancing agents for Alzheimer's disease. *Neurotherapeutics* **2008**, *5* (3), 458–469.

(41) Mitchell, E.S.; Neumaier, J.F. 5-HT_6 receptors: a novel target for cognitive enhancement. *Pharmacol Ther*. **2005**, *8* (3), 320-333.

(42) Study evaluating the safety and efficacy of PF-05212377 or placebo in subjects with Alzheimer's disease with existing neuropsychiatric symptoms on donepezil. Nct01712074, https://clinicaltrials.gov/ct2/show/NCT01712074, accessed 16 July 2015.

(43) Nirogi, R.; Jayarajan, P.; Abraham, R.; Kandikere, V. A safe, potent, selective, brain penetrant and orally active 5-HT_6 receptor antagonist SUVN-502. *12th Int Conf Alzheimer's Dis Relat Disord (ICAD)* **2009**, Abst P2–264.

(44) Lavrovsky, Y.; Ivachtchenko, A.V.; Morozova, M.; Salimov, R.M.; Kasey, V. Preclinical and early clinical studies of AVN-101, a novel balanced molecule for the treatment of Alzheimer's disease. *Alzheimer's & Dementia* **2010**, *6* (4), S583.

(45) Okun, I.; Tkachenko, S.E.; Khvat, A.; Mitkin, O.; Kazey, V.; Ivachtchenko, A.V. From anti-allergic to anti-Alzheimer's: molecular pharmacology of Dimebon. *Curr Alzheimer Res.* **2010**, *7*, 97–112.

(46) Cyprotex S9 stability assay. http://www.cyprotex.com/admepk/in-vitro-metabolism/s9-stability, accessed 16 July 2015.

(47) Merck Millipore. http://www.millipore.com, accessed 16 July 2015.

(48) Vivid® CYP450 screening kits protocol. https://tools.lifetechnologies.com/downloads/O-13873-r1_US_0405.pdf, accessed 16 July 2015.

(49) Guide for the Care and Use of Laboratory Animals. 1996, National Academy Press, Washington, DC.

(50) Ivashchenko, A.A.; Ivachtchenko, A.V.; Savchuk, N.P. 3-sulfonyl-pyrazolo[1,5-a] pyrimidines / antagonists of serotonin 5-HT_6 receptors, methods for the production and the use thereof. Patent RU2369600, **2009**.

(51) Ivachtchenko, A.V.; Golovina, E.S.; Kadieva, M.G.; Koryakova, A.G.; Mitkin, O.D.; Tkachenko, S.E.; Kysil, V.M.; Okun, I. 2-Substituted 5,6-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-a]pyrimidines: New series of highly potent and specific serotonin 5-HT6 receptor antagonists. *Europ J Med Chem.* **2011**, *46*, 1189–1197.

(52) Ivachtchenko, A.V.; Kadieva, M.G.; Golovina, E.S.; Koryakova, A.G.; Kuznecova, I.V.; Mitkin, O.D. 3-Arylsulfonylpyrazolo[1,5-a]pyrimidines as an antagonist of serotonin 5-HT_6 receptors. In: *Chemistry and medicine for the 70th anniversary of academician Yunusov M. S., Abstr. VIII All-Russ. Sci. Conf. with intern. Participation* **2010**, Inst. Organic Chem. Ufa Sci. Centre RAS, Russia, Ufa.

(53) Ivachtchenko, A.V.; Ivanenkov, Y.A. Small Molecule 5-HT_6R Ligands: A Comprehensive Insight into their Selectivity and Activity. *Curr Bioact Compd.* **2013**, *9*, 64–100.

(54) Arnt, J.; Bang-Andersen, B.; Grayson, B.; Bymaster, F.P.; Cohen, M.P.; DeLapp, N.W.; Giethlen, B.; Kreilgaard, M.; McKinzie, D.L.; Neill, J.C.; Nelson, D.L.; Nielsen, S.M.; Poulsen, M.N.; Schaus, J.M.; Witten, L.M. Lu AE58054, a 5-HT_6 antagonist, reverses cognitive impairment induced by subchronic phencyclidine in a novel object recognition test in rats. *Intern J Neuropsychopharmacol.* **2010**, *13*, 1021–1033.

(55) Chuang, A.T.; Foley, A.; Pugh, P.L.; Sunter, D.; Tong, X.; Regan, C.; Dawson, L.A.; Medhurst, A.D.; Upton, N. 5-HT_6 receptor antagonist SB-742457 as a novel cognitive enhancing agent for Alzheimer's disease. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association* **2006**, *2* (3), S631–S632.

(56) Ivachtchenko, A.V.; Kadieva, M.G.; Mitkin, O.D. The pharmacological activity of SB-742457. In: *Second Scientific Conference with International Participation "The successes and the synthesis of complex" on the 95th anniversary of the birth of Professor N. Prostakov, Peoples' Friendship University of Russia (RUDN)* **2012**, Moscow, Russia, abstracts.

(57) Hutcheson, J.D.; Setola, V.; Roth, B.L.; Merryman, W.D. Serotonin receptors and heart valve disease-it was meant 2B. *Pharmacol Ther* **2011**, *132* (2), 146–157.

(58) Polli, J.W.; Wring, S.A.; Humphreys, J.E.; Huang, L.; Morgan, J.B.; Webster, L.O.; Serabjit-Singh, C.S. Rational use of *in vitro* P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther.* **2001**, *299*, 620–628.

(59) Bromidge, S.M.; Brown, A.M.; Clarke, S.E.; Dodgson, K.; Gager, T.; Grassam, H.L.; Jeffrey, P.M.; Joiner, G.F.; King, F.D.; Middlemiss, D.N.; Moss, S.F.; Newman, H.; Riley, G.; Routledge, C.; Wyman, P. 5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046): a potent, selective, and orally bioavailable 5-HT6 receptor antagonist. *J Med Chem.* **1999**, *42*, 202−205.

(60) Nirogi, R.; Shinde, A.; Daulatabad, A.; Kambhampati, R.; Gudla, P.; Shaik, M.; Gampa, M.; Balasubramaniam, S.; Gangadasari, P.; Reballi, V.; Badange, R.; Bojja, K.; Subramanian, R.; Bhyrapuneni, G.; Muddana, N.; Jayarajan, P. Design, synthesis, and

pharmacological evaluation of piperidin-4-yl amino aryl sulfonamides: novel, potent, selective, orally active, and brain penetrant 5-HT_6 receptor antagonists. *J Med Chem.* **2012**, *55*, 9255−9269.

(61) Bromidge, S.M.; Clarke, S.E.; Gager, T.; Griffith, K.; Jeffrey, P.; Jennings, A.J.; Joiner, G.F.; King, F.D.; Lovell, P.J.; Moss, S.F.; Newman, H.; Riley, G.; Rogers, D.; Routledge, C.; Serafinowska, H.; Smith, D.R. Phenyl benzenesulfonamides are novel and selective 5-HT_6 antagonists: identification of *N*-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-piperazin-1-ylbenzenesulfonamide (SB-357134). *Bioorg Med Chem Lett.* **2001**, *11*, 55−58.

(62) Riemer, C.; Borroni, E.; Levet-Trafit, B.; Martin, J.R.; Poli, S.; Porter, R.H.P.; Bos, M. Influence of the 5-HT_6 receptor on acetylcholine release in the cortex: pharmacological characterization of 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, a potent and selective 5-HT6 receptor antagonist. *J Med Chem.* **2003**, *46*, 1273−1276.

(63) de Bruin, N.M.; Prickaerts, J.; van Loevezijn, A.; Venhorst, J.; de Groote, L.; Houba, P.; Reneerkens, O.; Akkerman, S.; Kruse, C.G. Two novel 5-HT_6 receptor antagonists ameliorate scopolamine-induced memory deficits in the object recognition and object location tasks in Wistar rats. *Neurobiol Learn Mem.* **2011**, *96*, 392−402.

(64) Ahmed, M.; Briggs, M.A.; Bromidge, S.M.; Buck, T.; Campbell, L.; Deeks, N.J.; Garner, A.; Gordon, L.; Hamprecht, D.W.; Holland, V.; Johnson, C.N.; Medhurst, A.D.; Mitchell, D.J.; Moss, S.F.; Powles, J.; Seal, J.T.; Stean, T.O.; Stemp, G.; Thompson, M.; Trail, B.; Upton, N.; Winborn, K.; Witty, D.R. Bicyclic heteroarylpiperazines as selective brain penetrant 5-HT_6 receptor antagonists. *Bioorg Med Chem Lett.* **2005**, *15*, 4867−4871

(65) Nirogi, R.V.; Badange, R.; Kambhampati, R.; Chindhe, A.; Deshpande, A.D.; Tiriveedhi, V.; Kandikere, V.; Muddana, N.; Abraham, R.; Khagga, M. Design, synthesis and pharmacological evaluation of 4-(piperazin-1-ylmethyl)-*N*1-arylsulfonyl indole derivatives as 5-HT_6 receptor ligands. *Bioorg Med Chem Lett.* **2012**, *22*, 7431−7435.

(66) Henderson, A.J.; Guzzo, P.R.; Ghosh, A.; Kaur, J.; Koo, J.M.; Nacro, K.; Panduga, S.; Pathak, R.; Shimpukade, B.; Tan, V.; Xiang, K.; Wierschke, J.D.; Isherwood, M.L. Epiminocyclohepta[b]indole analogs as 5-HT_6 antagonists. *Bioorg Med Chem Lett.* **2012**, *22*, 1494−1498.

(67) Tripathy, R.; McHugh, R.J.; Bacon, E.R.; Salvino, J.M.; Morton, G.C.; Aimone, L.D.; Huang, Z.; Mathiasen, J.R.; DiCamillo, A.; Huffman, M.J.; McKenna, B.A.; Kopec, K.; Lu, L.D.; Qian, J.; Angeles, T.S.; Connors, T.; Spais, C.; Holskin, B.; Duzic, E.; Schaffhauser, H.; Rossé, G.C. Discovery of 7-arylsulfonyl-1,2,3,4,4a,9a-hexahydrobenzo[4,5]furo[2,3-c]pyridines: identification of a potent and selective 5-HT_6 receptor antagonist showing activity in rat social recognition test. *Bioorg Med Chem Lett.* **2012**, *22*, 1421−1426.

(68) Singer, J.M.; Wilson, M.W.; Johnson, P.D.; Graham, S.R.; Cooke, L.W.; Roof, R.L.; Boxer, P.A.; Gold, L.H.; Meltzer, L.T.; Janssen, A.; Roush, N.; Campbell, J.E.; Su, T.Z.; Hurst, S.I.; Stoner, C.L.; Schwarz, J.B. Synthesis and SAR of tolylamine 5-HT_6 antagonists. *Bioorg Med Chem Lett.* **2009**, *19*, 2409−2412.

(69) Arnt, J.; Bang-Andersen, B.; Grayson, B.; Bymaster, F.P.; Cohen, M.P.; DeLapp, N.W.; Giethlen, B.; Kreilgaard, M.; McKinzie, D.L.; Neill, J.C.; Nelson, D.L.; Nielsen, S.M.; Poulsen, M.N.; Schaus, J.M.; Witten, L.M. Lu AE58054, a 5-HT_6 antagonist, reverses cognitive impairment induced by subchronic phencyclidine in a novel object recognition test in rats. *Int J Neuropsychopharmacol.* **2010***, 13*, 1021−1033.

(70) Sang, Z.; Qiang, X.; Li, Y.; Yuan, W.; Liu, Q.; Shi, Y.; Ang, .W.; Luo, Y.; Tan, Z.; Deng, Y. Design, synthesis and evaluation of scutellarein-O-alkylamines as multifunctional agents for the treatment of Alzheimer's disease. *Eur J Med Chem.* **2015**, *94*, 348–366.

(71) Liu, M.Y.; Wang, S.; Yao, W.F.; Zhang, Z.J.; Zhong, X.; Sha, L.; He, M.; Zheng, Z.H.; Wei, M.J. Memantine improves spatial learning and memory impairments by regulating NGF signaling in APP/PS1 transgenic mice. *Neuroscience* **2014**, *25*, 141–151.

(72) Spiazzi, C.C.; Soares, B.M.; Izaguirry, P.A.; Vargas, M.L.; Zanchi, M.M.; Pavin, F.N.; Affeldt, F.R.; Lüdtke S.D, Prigol, M.; Santos, F.W. Selenofuranoside ameliorates memory loss in Alzheimer-like sporadic dementia: AChE activity, oxidative stress, and inflammation involvement. *Oxid Med Cell Longev.* **2015**, 976908.

(73) Chao, F.; Zhang, L.; Luo, Y.; Xiao, Q.; Lv, F.; He, Q.; Zhou, C.; Zhang, Y.; Jiang, L.; Jiang, R.; Gu, H.; Tang, Y. Running Exercise Reduces Myelinated Fiber Loss in the Dentate Gyrus of the Hippocampus in APP/PS1 Transgenic Mice. *Curr Alzheimer Res.* **2015**, *12* (4), 77–383.

(74) Bryson, H.M.; Benfield, P. Donepezil. *Drugs Aging* **1997**, *10* (3), 234–239.

(75) Bourson, A.; Boess, F.G.; Bos, M.; Sleight, A.J. Involvement of 5-HT_6 receptors in nigro-striatal function in rodents. *Br J Pharmacol.* **1998**, *125*, 1562–1566.

(76) Bristow, L.J.; Collinson, N.; Cook, G.P.; Curtis, N.; Freedman, S.B.; Kulagowski, J.J.; Leeson, P.D.; Patel, S.; Ragan, C.I.; Ridgill, M.; Saywell, K.L.; Tricklebank, M.D. L-745,870, a subtype selective dopamine D4 receptor antagonist, does not exhibit a neuroleptic-like profile in rodent behavioral tests. *J Pharmacol Exp Ther.* **1997**, *283* (3), 1256–1263.

(77) Lesem, M. A randomized, placebo-controlled phase IIa trial of SGS518 for treating cognitive impairment associated with schizophrenia. *11th Int Congr Schizophr Res.* **2007**, Abstract.

(78) Efficacy study exploring the effect of Lu AE58054 as augmentation therapy in patients with schizophrenia. NCT00810667, https://clinicaltrials.gov/ct2/show/NCT00810667, accessed 16 July 2015.

(79) Lu AE58054 in patients with mild-moderate Alzheimer's disease treated with donepezil (STARBEAM). NCT02006641, https://clinicaltrials.gov/ct2/show/NCT02006641, accessed 16 July 2015.

(80) Study of Lu AE58054 in patients with mild - moderate Alzheimer's disease treated with donepezil (STARSHINE). NCT01955161, https://clinicaltrials.gov/ct2/show/NCT01955161, accessed 16 July 2015.

(81) Lu AE58054 in patients with mild to moderate Alzheimer's disease treated with an acetylcholinesterase inhibitor (STARBRIGHT). NCT02006654, https://clinicaltrials.gov/ct2/show/NCT02006654, accessed 16 July 2015.

(82) Long-term safety and tolerability of Lu AE58054 as adjunctive treatment to donepezil in patients with mild-moderate Alzheimer's disease (STAR Extension). NCT02079246, https://clinicaltrials.gov/ct2/show/NCT02079246, accessed 16 July 2015.

(83) Lu AE58054 added to donepezil for the treatment for moderate Alzheimer's disease. NCT01019421, https://clinicaltrials.gov/ct2/show/NCT01019421, accessed 16 July 2015.

(84) Wilkinson, D.; Windfeld, K.; Colding-Jørgensen, E. Safety and efficacy of idalopirdine, a 5-HT_6 receptor antagonist, in patients with moderate Alzheimer's disease (LADDER): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol.* **2014**, *13* (11), 1092–1099.

(85) Ahmed, M.; Johnson, C.N.; Jones, M.C.; Macdonald, G.J.; Moss, S.F.; Thompson, M.; Wade, C.E.; Witty, D. Quinoline derivatives and their use as 5-HT_6 ligands. Patent WO2003080580, **2004**.

(86) Johnson, C.N.; Witty, D. Quinoline compounds and pharmaceutical compositions containing them. Patent WO2005026125, **2005**.

(87) Gladwin, A.E. A polymorphic form of 3-phenylsulfonyl-8-piperazin-1-yl-quinoline. Patent WO 2005040124, **2005**.

(88) Chuang, T.T.; Hunter, A.J.; Virley, D.J. Combinations comprising 3-phenylsulfonyl-8-piperazinyl-1yl-quinoline. Patent WO2009074607, **2009**.

(89) Arnt, J.; Bang-Andersen, B.; Dias, R.; Bøgesø, K.P. Strategies for pharmacotherapy of schizophrenia. *Drugs of the Future* **2008**, *33* (9), 777–791.

(90) Maher-Edwards, G.; Dixon, R.; Hunter, J.; Gold, M.; Hopton, G.; Jacobs, G.; Hunter, J.; Williams, P. SB-742457 and donepezil in Alzheimer disease: a randomized, placebo-controlled study. *Int J Geriatr Psychiatry* **2010**, *26* (5), 536–544.

(91) Maher-Edwards, G.; Zvartau-Hind, M.; Hunter, A.J.; Gold, M.; Hopton, G.; Jacobs, G.; Davy, M.; Williams, P. Double-blind, controlled phase II study of a 5-HT_6 receptor antagonist, SB-742457, in Alzheimer's disease. *Curr Alzheimer Res.* **2010**, *7* (5), 374–385.

(92) Maher-Edwards, G.; Zvartau-Hind, M.; Hunter, A.J.; Gold, M.; Hopton, G.; Jacobs, G.; Davy, M.; Williams, P. Double-blind, controlled phase II study of a 5-HT_6 receptor antagonist, SB-742457, in Alzheimer's disease. *Curr Alzheimer Res.* **2010**, *7* (5), 374–385.

(93) Axovant RVT-101, http://www.axovant.com/rvt-101/, accessed 16 July 2015; ClinicalTrials.gov Identifier: NCT02585934.

(94) Morozova, M.A.; Lepilkina, T.A.; Rupchev, G.E.; Beniashvily, A.G.; Burminskiy, D.S.; Potanin, S.S.; Bondarenko, E.V.; Kazey, V.I.; Lavrovsky, Y.; Ivachtchenko, A.V. Add-on clinical effects of selective antagonist of $5HT_6$ receptors AVN211 (CD0080173) in patients with schizophrenia stabilized on antipsychotic treatment: pilot study. *CNS Spectr.* **2013**, *19* (4), 316–323.

(95) Avineuro completed phase II clinical study of AVN-211, a selective 5-HT_6 receptor antagonist. http://www.biospace.com/News/avineuro-completed-phase-ii-clinical-study-of-avn/384442/source=MoreNews, accessed 16 July 2015.

(96) Gravius, A.; Laszy, J.; Pietraszek, M.; Sághy, K.; Nagel, J.; Chambon, C.; Wegener, N.; Valastro, B.; Danysz, W.; Gyertyán, I. Effects of 5-HT6 antagonists, Ro-4368554 and SB-258585, in tests used for the detection of cognitive enhancement and antipsychotic-like activity. *Behav Pharmacol*. **2011**, *22* (2), 122-135.

(97) Russell, M.G.; Dias, R. Memories are made of this (perhaps): a review of serotonin 5-HT_6 receptor ligands and their biological functions. *Curr Top Med Chem*. **2002**, *2* (6), 643-654.

(98) Lindner, M.D.; Hodges, D.B.Jr.; Hogan, J.B.; Orie, A.F.; Corsa, J.A.; Barten, D.M.; Polson, C.; Robertson, B.J.; Guss, V.L.; Gillman, K.W.; Starrett, J.E.Jr.; Gribkoff, V.K. An assessment of the effects of serotonin 6 (5-HT_6) receptor antagonists in rodent models of learning. *J Pharmacol Exp Ther*. **2003**, *307* (2), 682-691.

(99) Schreiber, R.; Vivian, J.; Hedley, L.; Szczepanski, K.; Secchi, R.L.; Zuzow, M. Effects of the novel 5-HT_6 receptor antagonist, Ro4368554, in rat models for cognition and sensorimotor gating. *Eur Neuropsychopharmacol*. **2007**, *17*, 277–288.

(100) Marcos, B.; Chuang, T.T.; Gil-Bea, F.J.; Ramirez, M.J. Effects of 5-HT_6 receptor antagonism and cholinesterase inhibition in models of cognitive impairment in the rat. *Br J Pharmacol*. **2008**, *155*, 434–440.

(101) Pouzet, B.; Didriksen, M.; Arnt, J. Effects of the 5-HT_6 receptor antagonist, SB-271046, in animal models for schizophrenia. *Pharmacol Biochem Behav*. **2002**, *71*, 635–643.

(102) Lieben, C.K.; Blokland, A.; Sik, A.; Sung, E.; Van, N.P.; Schreiber, R. The selective 5-HT_6 receptor antagonist, Ro4368554, restores memory performance in cholinergic and serotonergic models of memory deficiency in the rat. *Neuropsychopharmacology* **2005**, *30*, 2169–2179.

(103) Foley, A.G.; Murphy, K.J.; Hirst, W.D.; Gallagher, H.C.; Hagan, J.J.; Upton, N. The 5-HT_6 receptor antagonist SB-271046 reverses scopolamine-disrupted consolidation of a passive avoidance task and ameliorates spatial task deficits in aged rats. *Neuropsychopharmacology* **2004**, *29*, 93–100.

(104) Schreiber, R.; Vivian, J.; Hedley, L.; Szczepanski, K.; Secchi, R.L.; Zuzow, M. Effects of the novel 5-HT_6 receptor antagonist, Ro4368554, in rat models for cognition and sensorimotor gating. *Eur Neuropsychopharmacol*. **2007**, *17*, 277–288

(105) Marcos, B.; Chuang, T.T.; Gil-Bea, F.J.; Ramirez, M.J. Effects of 5-HT_6 receptor antagonism and cholinesterase inhibition in models of cognitive impairment in the rat. *Br J Pharmacol*. **2008**, *155*, 434–440.